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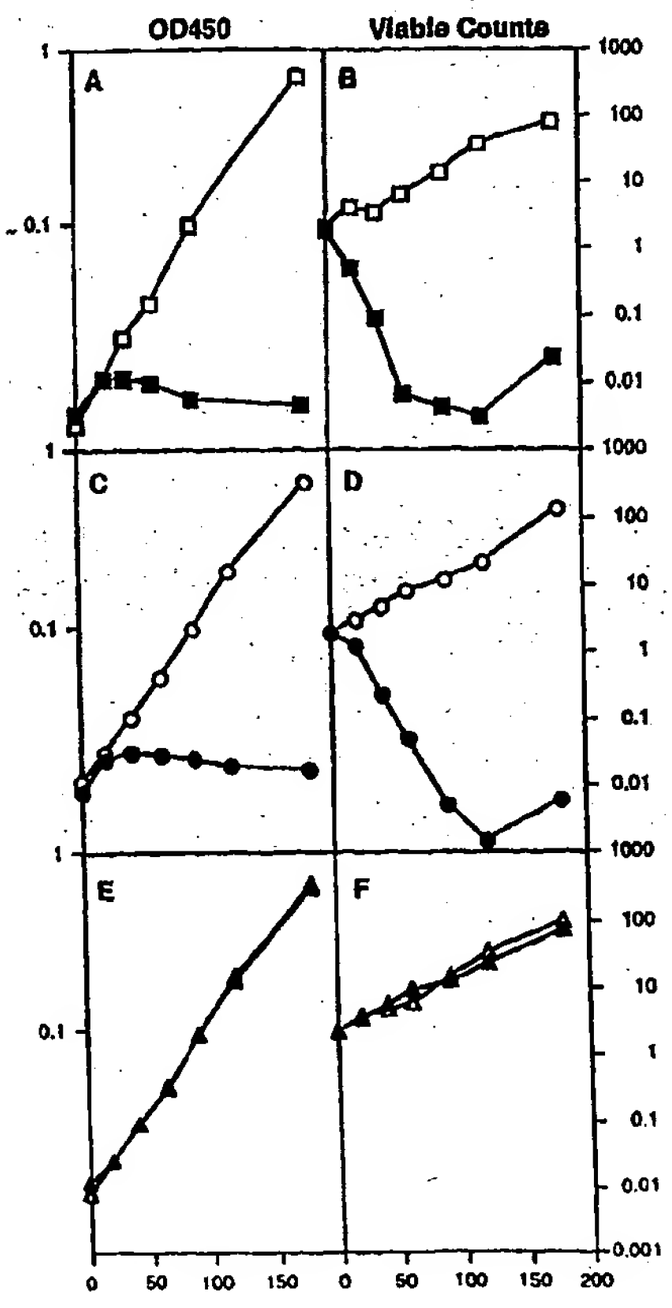
(54) Title: CYTOTOXIN–BASED BIOLOGICAL CONTAINMENT

(57) Abstract

Method of conditionally controlling the survivability of a recombinant cell population and of containing such cells to an environment or containing replicons to a host cell is based on the use of proteic killer systems including the *E. coli relBE* locus and similar systems found in Gram–negative and Gram–positive bacteria and *Archae*. Such system are generally based on a cytotoxin polypeptide and an antitoxin or antidote polypeptide that in contrast to the cytotoxin is degradable by proteases. The recombinant cells are useful as vaccines, pollulant degrading organisms or as biogical pest control organisms e.g. expressing *B. thuringiensis* crystalline proteins.



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CYTOTOXIN-BASED BIOLOGICAL CONTAINMENT

FIELD OF THE INVENTION

The present invention relates to the field of biologically containing genetically modified microorganisms in a particular environment and vectors to a particular host cell. Specifically, there is provided recombinant vectors and cells containing a proteic killer system based on the *E. coli* RelE polypeptide and functional equivalents of this cytotoxin and method of containing replicons and cells, respectively to particular host cells or particular environments, respectively.

TECHNICAL BACKGROUND AND PRIOR ART

The increasing application of recombinant DNA technology to engineer novel microorganism which are industrially useful have caused concerns in the general public over the potential risks involved. These concerns are primarily related to the potential harm to humans and to undesirable and/or uncontrollable ecological consequences upon deliberate or unintentional release of such genetically engineered microorganisms (GEMs) into the environment. These concerns have led to the establishment of official guidelines for the safe handling of GEMs in laboratories and production facilities where such organisms are applied. Up till now, such guidelines have primarily been directed to measures of physically containing GEMs in laboratories and production facilities with the aim of reducing the likelihood that workers in such facilities were contaminated, or that the GEMs were to escape from their primary physical environment, such as a fermentation vessel.

It is presently being recognized that the level of safety in the handling of GEMs can be increased by combining physical containment measures with biological containment measures to reduce the possibility of the survival of the genetically engineered organisms if they were to escape from their primary environment.

Lately, however, concerns have become increasingly focused on potential risks related to deliberate release of GEMs to the outer environment and to the use of GEMs as live vaccines. In this connection there is a strongly felt need to have biological containment systems which subsequent to the environmental release of the GEMs or their administration as vaccines to a human or an animal body, effectively kill the released organisms in a controlled manner or which limit the function of the released GEMs to an extent where such GEMs are placed at a significant competitive disadvantage whereby they will eventually be ousted by the natural microflora of the environment to which they are released.

The first systems of biological containment were based on the use of "safe" cloning vectors and debilitated host bacteria. As examples, it has been suggested to select vectors which lack transfer functions or which naturally have a very narrow host range. Examples of debilitated host bacteria are *E. coli* mutants having an obligate requirement for exogenous nutrients not present or present in low concentrations outside the primary environment of the GEMs.

Other suggested biological containment systems have been based on mechanisms whereby the vector is restricted to the GEMs e.g. by using a plasmid vector with a nonsense mutation in a gene, the expression of which is indispensable for plasmid replication or a suppressor mutation in the chromosome, said mutation blocking translational read-through of the message of the gene. A further approach is to maintain the rDNA stably in the host by integrating it into the chromosomes of the GEMs.

Recently, an alternative biological containment strategy has been developed in which a recombinant vector is endowed with a gene encoding a cell killing function which gene is under the control of a promoter only being expressed under certain environmental conditions, such as conditions prevailing in an environment outside the primary environment of the GEMs, or when the vector is unintentionally transferred to a secondary host, or the expression of which is stochastically induced. By using incorporation in a GEM of such a cell killing function and selecting appropriate regulatory sequences, vectors can be constructed which are contained in the primary host cell and/or in a primary physical environment. A cell killing function as defined herein may also be referred to as an active biological containment factor.

If a stochastically induced mechanism of expression regulation is selected for such a biological containment system, a population of GEMs containing the system will, upon release to the outer environment or if used as a live vaccine, be subjected to a random cell killing which will lead to an increase of the doubling time of the host cell population or eventually to the disappearance of the organisms.

The above-mentioned genes encoding cell killing functions are also frequently referred to as "suicide" genes, and biological containment systems based upon the use of such genes, the expression of which are regulated as defined above, are commonly described as conditional lethal systems or "suicide" systems. Up till now, several such cell killing functions have been found in bacterial chromosomes and in prokaryotic plasmids. Examples of chromosomal genes having cell killing functions are the *gef* (Poulsen et al., 1990) and *relF* (Bech et al., 1985) genes from *E. coli* $_{K\text{-}12}$. Examples of plasmid encoded suicide genes are *hok* and *flmA* (Gerdes et al., 1986) genes isolated from plasmids R1 and F, respectively, the *snr*B gene also isolated from plasmid F (Akimoto et al., 1986) and the *pnd* gene isolated from plasmids R16 and R483 (Sakikawa et al., 1989 and Ono et al., 1987). Common features of these genes are that they are transcribed constitutively, regulated at a post-transcriptional level, and that they all encode small toxic proteins of about 50 amino acids and that their translation is controlled by antisense RNA. The application of the *hok* gene in a biological containment system has been disclosed in WO 87/05932.

An alternative biological containment system is disclosed in WO 95/10614 which is based on the use of genes, the expression of which in a cell where the gene is inserted, results in the formation of mature forms of exoenzymes which are hydrolytically active in the cytoplasm of the cell and which can not be transported over the cell membrane. When such enzymes are expressed, the normal function of the cell becomes limited to an extent whereby the competitiveness, and hence the survival, of a population of such cells is reduced.

The stable maintenance of low copy-number plasmids in bacteria is secured by a number of plasmid-borne gene systems, one of which is based on killing of plasmid-free cells (also termed post-segregational killing). This regulated killing is based on a toxin-antidote principle, i.e. a two-component system comprising a stable toxin and an un-

stable antidote for the toxin. One such system, which is referred to as a proteic killer gene system is based on protein toxins and protein antidotes (reviewed by Jensen and Gerdes, 1995). The natural function of such systems is to provide stable maintenance of plasmids and it has not been suggested previously to utilize the systems as the basis for confining GEMs to a particular environment.

The *E. coli relB* operon encodes three genes, *relB*, *relE* and *relF* (Bech et al., 1985). It has now been found that *relE* encodes a cytotoxin whose overproduction is lethal to host cells and that the *relB* gene encodes an antitoxin that prevents the lethal action of RelE. When present on a plasmid, the *relBE* operon was able to stabilize the inheritance of a mini-R1 test plasmid. It was also found that *relBE* homologous gene systems are found in a wide variety of Gram-negative and Gram-positive bacteria and in *Archae*.

These results show that the *relBE* genes constitute a new ubiquitously occurring family of gene systems that belongs to the proteic plasmid stabilization systems.

These findings has opened up for an alternative, highly effective and versatile biological containment system as it is described in the following. Importantly, it has been discovered that such a system involves the significant advantage that the frequency of spontaneously occurring mutants of microorganisms that have become resistant to the lethal effect of these cytotoxins is very low. This implies that this biological containment system is very safe.

SUMMARY OF THE INVENTION

Accordingly, the invention pertains in a first aspect to a method of conditionally controlling the survivability of a recombinant microbial cell population, the method comprising (i) providing in the cells of said population a gene coding for a cytotoxic first kind of polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally equivalent polypeptide (said genes collectively being designated herein as the *relE* gene family), said gene is expressible in the cells of the population and, operably linked to the gene,

a regulatable regulatory DNA sequence and (ii) cultivating the cell population under conditions where the *relE* gene or the gene coding for a functionally equivalent polypeptide is expressed, the expression leading to an at least partial killing of the cell population.

In a further aspect there is provided a method of confining an extrachromosomal replicon to a microbial cell population, the method comprising the steps of

(i) isolating a microbial cell naturally containing a gene belonging to the *relE* gene family coding for a first kind of polypeptide that, when it is expressed in the cell, acts as a toxin for the cell or, if the cell does not naturally contain a gene belonging to the *relE* gene family, introducing such a gene into the cell,

(ii) introducing into the cell the extrachromosomal replicon to be confined, said replicon containing a gene coding for a second kind of polypeptide that, by binding to the first kind of polypeptide, acts as an antitoxin for said first kind of polypeptide,

(iii) cultivating the cell under conditions where the genes coding for the first and the second kind of polypeptides are expressed, whereby a daughter cell that does not receive a copy of the extrachromosomal replicon is killed by the first kind of polypeptide being expressed in the absence of expression of the second kind of polypeptide.

In a still further aspect, the invention relates to a method of post-segregationally stabilizing a plasmid in a microbial host cell population, the method comprising the steps of

(i) recombinationally inserting into the plasmid (a) a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a functional equivalent thereof, said first kind of polypeptide having a toxin effect on the host cell and (b) a gene coding for a second kind of polypeptide that (1) is capable of acting as an antitoxin for first kind of polypeptide and (2) is capable of being degraded in the host cell at a higher rate than that at which the first kind of polypeptide is degraded,

(ii) cultivating the cell population under conditions where the genes coding for the first kind and second kind of polypeptides are expressed,

whereby a daughter cell that does not receive at least one copy of the plasmid is killed as a result of the faster degradation of the second kind of polypeptide.

In yet other aspects, the invention provides a recombinant microbial cell comprising a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, a gene coding for a functionally equivalent polypeptide hereof or a variant or derivative of any such polypeptide, said first kind of polypeptide having a toxic effect on the cell, subject to the limitation that when the cell is *E. coli*, the gene coding for the first kind of polypeptide is not derived from *E. coli*, and a composition comprising such cells.

The invention also pertains to several methods of containing cells or replicons including

(1) a method of limiting the survival of a cell population in a first or a second environment, which method comprises

(i) transforming the cells of said population with a gene coding for a cytotoxic polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide, the gene coding for the prophage P1 Doc polypeptide and a gene coding for a functionally equivalent polypeptide for anyone of said polypeptides, said gene is expressible in the cells of the population, and operably linked to the gene, a regulatory DNA sequence being regulatable by an environmental factor and which regulates the expression of said gene, and

(ii) cultivating the cell population under environmental conditions where the gene coding for the cytotoxic polypeptide is expressed, the expression leading to an at least partial killing of the cell population,

(2) a method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is naturally transferable to a second kind of cell, which method comprises providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides to an extent whereby the function of the cell is being limited, said first kind of cells having or being modified to have a chromosomal replicon comprising a regulatory nucleotide sequence the gene product of which inhibits the expression of said gene or the cell function-limiting effect of the polypeptide and thereby protects said first kind of cells, said regulatory gene being lacking in said second kind of cell, whereby, if a cell of the second kind receives said extrachromosomal recombinant replicon said gene is expressed and has a function-limiting effect on said second kind of cell, and

(3) a method of stochastically limiting in an environment the survival of a cell population, the method comprising transforming the cells thereof with a recombinant replicon containing a regulatably expressible gene which, when expressed in a cell, encodes a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides, the expression of said gene leading to formation of the polypeptide to an extent whereby the function of the cells is being limited, the expression of said gene is stochastically induced as a result of recombinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cells, inhibits expression of the gene coding for the polypeptide, said negatively functioning regulatory nucleotide sequence being contained in the recombinant replicon or in an other recombinant replicon present in cells of the population containing the replicon.

DETAILED DISCLOSURE OF THE INVENTION

One objective of the present invention is to provide a novel approach to conditionally controlling the survivability of a recombinant microbial cell population. This approach is based on the use of what is generally referred to as proteic killer systems which have been reviewed i.a. by Jensen et al., 1995. These systems consist of two components, a cytotoxin polypeptidé (also referred to herein as a first kind of polypeptide) and a corresponding antitoxin or antidote polypeptide (also referred to herein as a second kind of polypeptide) that by binding to the cytotoxic polypeptide inhibits the toxic effect hereof. A general characteristic of such proteic killer systems is that the antitoxin component in contrast to the toxin component is susceptible to protease degradation, resulting in a decay of the antitoxin polypeptide.

As used herein, the expression "microbial cell" includes any prokaryotic and eukaryotic cells as well as cells of *Archae* species. Thus this expression includes cells of bacterial species, fungal species, animal species including invertebrates, vertebrates, mammals, humans and insects, and plant cells.

Thus, in one aspect of the invention there is provided such a method of conditionally controlling the survivability of a recombinant microbial cell population that comprises as the first step, providing in the cells a gene coding for a cytotoxic first kind of polypeptide, which gene is selected from the gene coding for the *E. coli* K-12 RelE polypeptide and a gene coding for a structurally and functionally equivalent polypeptide and, operably linked to the gene, a regulatable regulatory DNA sequence.

Genes which are structurally and functionally equivalent to *relE* are herein collectively referred to as the *relE* gene family or as *relE* homologues. This group of genes including the *E. coli* plasmid P307 derived *relE* homologue encompasses genes the gene products of which have cytotoxic effects and which, relative to *E. coli* K-12 RelE, have at least 20% such as at least 30% e.g. at least 40% identical and conserved amino acids. The sequences listed in the below Table 1.5 are putative RelE homologues.

Whereas, in accordance with the invention, presently preferred recombinant microbial cells are prokaryotic cells such as Gram-negative and Gram-positive bacterial cells, it has been found that the survivability of other microbial cells such as *Archae*, yeast cells, fungal cells, animal cells including human cells and plant cells and replicons of such organisms can be conditionally controlled using the methods of the present invention.

In the present context, the expression "conditionally controlling" refers to a construction of the microbial cell which permits that the gene coding for the cytotoxic polypeptide can be expressed under certain pre-determined environmental conditions whereas under other such conditions, the gene is not expressed. Hence, the survivability of the microbial cells can be made dependent on certain pre-selected conditions.

In accordance with the invention, the survivability of microbial cells is controlled by the expression in the cells of a cytotoxic polypeptide selected from *E. coli* K-12 RelE polypeptide and a functionally equivalent polypeptide. As used herein, the term "cytotoxic" refers not only to a loss of the ability of microbial cells containing the toxin-encoding gene to remain viable as determined by the capability to propagate in media which, under identical environmental conditions, support unrestricted growth of similar cells not containing the toxin-encoding gene, but also to cells having, as a result of the expression of the polypeptide-encoding gene, a limited cell function, the latter expression denoting that the growth of a cell as manifested i.a. by the synthesis of new cell material and the rate of replication of the cell is decreased.

During the experimentation leading to the invention it was surprisingly found that a range of cytotoxic poplypeptides according to the invention have the effect that they inhibit translation of genes. This general effect of RelE polypeptides and functionally equivalent polypeptides appears to represent a hitherto undiscovered mechanim for controlling survivability of cells and thus for containment of such cells or replicons in accordance with the methods of the present invention.

Whereas the recognizable manifestation of such limited cell function may ultimately be cell death, it may also be a reduced cell growth appearing as a reduced rate of replication resulting in a reduced increase of cell numbers within a certain period of time as a

result of an increase of the lag phase and/or of the cell doubling time. Other manifestations may be a relatively increased requirement for one or more nutrient components or a relatively higher susceptibility to detrimental environmental factors such as sub-optimal temperatures or cell damages caused by toxic substances.

In the present context, the expression "a functionally equivalent polypeptide" refers to a polypeptide that has substantially the same effect on the survivability of microbial cells as the RelE polypeptide of *E. coli* K-12. As it is shown herein, a variety of Gram-positive and Gram-negative bacteria and *Archae* organisms comprise DNA sequences showing homology with the RelE polypeptide. To the extent gene products of structural homologues of the *relE* gene product show an effect on microbial cell survivability as it is defined above, they are encompassed by the present invention. It will also be appreciated that the term "functional equivalent" includes variants or derivatives of any of the above first kind of polypeptides, the sequences of which have been modified by substitution, deletion or addition of one or more amino acids and the gene product of which has retained at least part of the cytotoxic function of the gene product of the non-modified sequence.

In the above method, a regulatable regulatory DNA sequence is operably linked to the gene coding for the cytotoxic polypeptide. In accordance with the invention, such a regulatory sequence can be one with which the gene coding for the cytotoxic polypeptide is naturally associated or it can be a sequence with which the gene is not naturally associated. In the present context, the term "regulatory DNA sequence" is intended to indicate a DNA sequence which directly or indirectly regulates the expression of the gene coding for the cytotoxic polypeptide at the level of transcription or at the level of translation or at the level of protein function. The regulatory DNA sequence may thus be one, the function of which results in a suppression or inhibition of the activity of the regulatable promoter.

Such regulatory DNA sequences are referred to herein as "negatively functioning regulatory DNA sequences". One interesting example of such a regulatory DNA sequence is a sequence coding for a repressor substance which represses the expression of the gene coding for cytotoxically active polypeptide and which substance may, when a cell containing it is released to a human or an animal body or to the outer envi-

ronment where the substance is no longer being expressed, undergo a decay whereby the repression of expression of the cytotoxin-encoding gene is gradually reduced and eventually, when the decay of the repressor is completed, the repression is removed.

Another example of such a regulatory DNA sequence is a sequence encoding a polypeptide that acts as an antidote or antitoxin for the cytotoxic polypeptide. Such a sequence include the *relB* gene derived from the *relBE* operon of *E. coli* K-12 which is capable of binding to the RelE polypeptide and thereby inhibiting its effect. As also shown herein, sequences encoding such antitoxins can be found in Gram-negative and Gram-positive bacteria and in *Archae*. Such homologues of the *relB* sequence are encompassed by the present invention.

In preferred embodiments of the invention, the regulatory DNA sequence may be present in the cell in one or more recombinant replicons and it may be contained in the same replicon as that containing the cytotoxin-encoding gene or in a different recombinant replicon.

One way whereby the expression of the cell function-limiting cytotoxic polypeptide can be regulated is by providing in the cell a gene coding for the polypeptide, which gene is regulated at the level of transcription. The regulation at the level of transcription may be carried out in various ways including a regulation by means of a promoter, regulated by one or more factors. These factors may either be ones which by their presence ensure expression of the gene coding for polypeptide or may, alternatively, be factors which suppress the expression of the gene so that their absence causes the polypeptide to be expressed.

Factors regulating the activity of the promoter as defined above may be selected from a variety of factors. Thus, the expression of the gene encoding the polypeptide may be determined by the environmental conditions, by the physiological state of the cells, or by a cyclical or stochastic event. In the present context, the term "cyclical event" is understood to mean a cyclically recurrent event causing changes in certain factors known to be potentially useful in affecting the expression of genes such as temperature conditions, changes in light intensity or hormonal changes. The term "physiologi-

cal state of the cells" denotes factors such as cell density or the growth phase of cells.

In accordance with the invention, advantageous promoter regulating factors are readily regulatable factors including the presence or absence of a certain chemical substance in the environment or the physical conditions in the environment such as the prevailing temperature or other physical factors (e.g. the intensity of the light in the environment). Thus, it is possible to envisage containment systems as presently claimed, in which the gene coding for the cytotoxic polypeptide is expressed when a certain chemical substance present in a first environment such as the fermentation medium in which the cell is propagated, is not present in a second environment to which the cell is released, or when a factor required for the growth or survival of the cell is no longer present, or the factor is one which, when it is depleted or exhausted from an environment of the cell, has the desired effect, viz. that the gene is expressed.

The promoter regulating the transcription of the gene coding for the cytotoxic polypeptide can also become activated in a second environment of the cell by a chemical substance which is not present in a first environment of the cell, but which is present in the second environment in sufficient quantities to activate the promoter. Similarly, the promoter may be a promoter which is activated by a shift in temperature, such as a shift from a higher temperature in a first environment as e.g. a fermentation vessel, to a lower temperature prevailing in an outside second environment, or the intensity of light, in that the promoter may be one which is activated in the presence of light of sufficient intensity, but is inactive in the darkness prevailing in a first environment such as a fermentation vessel.

Where microbial cells as defined herein are cells that are to be released to the outer environment in a controlled manner, e.g. to a restricted area of land or to the intestinal tract of a human or an animal, the regulatable promoter may be one which is regulated chemically, i.e. by the presence or absence of a certain chemical substance in the environment of the cells as it has been explained above.

However, the regulatable promoter is advantageously a promoter which is activated cyclically, e.g. by changes of the temperature, or by a stochastic event. The term

"stochastic event" as used herein is intended to denote an event which occurs at random at a certain frequency per cell per generation or frequency per unit time which, in accordance with the invention, may result in a limitation of the function of the cells in which the activation of expression of the cytotoxic polypeptide occurs, optionally to an extent which leads to the death of the cells. The stochastic event may be occasioned by periodic inversions of the region carrying the promoter or by recombinational excision of a recombinationally excisable negatively functioning regulatory DNA sequence as defined above.

It should be noted that in order to ensure a general applicability of the present invention, the promoter used to initiate transcription of the gene coding for the toxic polypeptide is preferably a promoter which is capable of causing expression of said gene in a wide range of cells.

In case of regulatable transcription of the polypeptide, the regulatory DNA sequence may e.g. be a promoter isolated from bacterial operons involved in the biosynthesis of amino acids or from bacterial genes, the transcription of which is activated late in the stationary growth phase or from bacterial genes involved in the synthesis of cell surface structures such as fimbriae. Examples of suitable promoters include the *E. coli trp* promoter which becomes activated in the absence of tryptophan, the bacteriophage λ P_R and P_L promoters controlled by temperature sensitive regulatory DNA sequences, the *Bacillus subtilis* sporulation gene promoters which are activated during sporulation, and the *E. coli* and *Salmonella* fimbriae gene promoters which are activated stochastically.

In case of chemically regulatable promoters, the chemical substance, the presence or absence of which determines the activation of the promoter, is suitably selected from carbon or nitrogen sources, metabolites, amino acids, nucleosides, purine or pyrimidine bases or metal ions. When the chemical substance is one which, when present, suppresses promoter activity, it is preferably a substance which rarely occurs in the natural environment in such concentrations that the promoter would not be activated when the cell is released to the natural environment. One example of such a promoter is the *trp* promoter which is repressed in the presence of tryptophan in the environment of the cell, but which is derepressed in the absence of sufficient amounts of

tryptophan in the environment. A containment system according to the invention using the *trp* promoter or another promoter being regulated in the same manner, might therefore comprise an amount of tryptophan in a first environment, such as a fermentation vessel, which is sufficient to repress the promoter in such an environment, the promoter, however, being derepressed when the cell is released from the first environment to a second environment, e.g. the outer environment which usually contains very low amounts of tryptophan or no tryptophan at all.

It is also possible to select a promoter that is regulated by the absence or presence of one or more compounds in exudates of plants colonized with a recombinant organism according to invention.

In this context, another useful promoter is an arabinose inducible promoter including that contained in the plasmid pBAD (Guzman et al., 1995). Without arabinose added to the growth medium, the pBAD promoter is completely turned off. However, in the presence of arabinose, strong transcription is induced. This particular promoter is repressible by the addition of glucose to the growth medium. Thus, by the addition of glucose, transcription from pBAD can be rapidly and efficiently turned off. The glucose repression effect is epistatic to the inducer effect by arabinose. Hence, if cells with a pBAD-carrying plasmid are grown in a medium containing both arabinose and glucose, the promoter is not induced. However, if cell growth depletes the medium for glucose, then the promoter will be induced. Therefore, such a plasmid is suitable for the conditional turning on and off the expression of gene, in particular toxin-encoding genes as described herein.

Accordingly, in one embodiment of the invention the method is used to contain microbial cells wherein the promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound whereby, when the first kind of compound is depleted from the medium, the promoter is induced by the second kind of compound.

Another example of a regulatable promoter, the activation of which is determined by a chemical substance is the *lac* promoter which is inducible by e.g. isopropyl-ß-D-thiogalactopyranoside (IPTG).

As mentioned above, the regulatable promoter may be a promoter, the activity of which is determined by the temperature prevailing in the environment of a cell containing the gene coding for the cell function-limiting cytotoxin and a regulatable promoter regulating the expression of the gene. In such a case, the regulation of the promoter is advantageously obtained by the presence in the cell of a gene coding for a temperature sensitive repressor for the promoter. As one typical example, the λ promoters including those mentioned above may be regulated by a temperature sensitive λ cI repressor.

Promoters which are activated stochastically by periodic inversions of the promoter region (in the present context, such promoters are also termed as "invertible promoters" and "inversional switch promoters") and which are useful for the purposes of the present invention include as examples the *hin*, *cin* and *gin* promoters. One particularly useful invertible promoter is the *fimA* promoter which is one *E. coli* fimbriae promoter. The activation (inversional switch) of this promoter is regulated by the gene products of the two genes which for the present purposes is termed the "on" and the "off" genes, the on gene product inducing a switch from off (inactive) to on (active), and the *off* gene product inducing a switch from on to off. In a wild-type *E. coli* cell where the *fimA* gene and its associated promoter is present in one copy on the chromosome, the inversional switch occurs with a switching frequency of about one cell/1000 cells/generation. It is, however, possible to regulate the frequency of the inversional switch as required by regulating the dosage of expression of the *on* and *off* genes. This is e.g. effected by means of suitable promoters transcribing into the on and off genes. The frequency of transcription initiation by these promoters will then determine the relative dosage levels of the *on* and *off* gene products being formed.

In accordance with the invention, one particular method of stochastically regulating the expression of the gene coding for the toxic polypeptide is the induction of the gene expression as a result of recombinational excision of an excisable negatively functioning regulatory DNA sequence which, while present in the cell, inhibits expression of the gene. In the present context, the term "recombinational excision" refers to the result of a naturally occurring phenomenon of genetic recombination (cross-over) whereby DNA sequences in replicons, in a controlled process, pair, brake and rejoin to

form recombinant replicons by the sequential action of enzymes acting on the DNA. The frequency of recombinational events in a cell depends i.a. on the degree of homology between paired complementary nucleotide sequences and on the length of the complementary sequences. Thus, it has been shown that about 50 base pairs of homology may be required to obtain recombination in a bacterial cell.

When a negatively regulatory DNA sequence is inserted between directly repeated nucleotide sequences of a sufficient length in a recombinationally proficient cell which, in accordance with the invention contains a gene coding for the toxic polypeptide, recombination between the repeats results in the recombinational excision of the negatively regulatory DNA sequence permitting the gene to be expressed.

Accordingly, the phenomenon of recombinational excision implies that a DNA subsequence, i.e. the negatively regulatory DNA sequence, is excised from a longer DNA sequence through a recombination event. In essence, the longer DNA sequence is cleaved on either side of the subsequence and the fresh ends are joined, leaving out the subsequence. Recombination occurs between sufficient homologous flanking nucleotide subsequences. Thus, with DNA of the general structure W-X-Y-X-Z, X being a repeated sequence and Y being a negatively regulatory DNA sequence, this could recombine to form W-X-Z, with the Y subsequence being excised.

As mentioned above, the frequency of the recombination can be determined by varying the lengths of the repeats and/or the distance between the repeats. Furthermore, the frequency may be varied by using repeat sequences of varying homologies. Thus, nucleotide sequence repeats being 100% homologous and having a size which does not impair recombination will result in a high recombination frequency and hence, in a high frequency of recombinational excision of the negatively regulatory sequence, whereas mismatches within complementary sequences will reduce the recombination frequency depending on the degree of mismatch. As an example, it has been found that 10% divergence between nucleotide sequence repeats may reduce the recombination frequency 40-fold.

Accordingly, the microbial cell comprising the gene coding for the cytotoxic polypeptide may, in accordance with the invention, be a cell containing a regulatory DNA se-

quence which is a recombinationally excisable negatively functioning regulatory DNA sequence being flanked by a first flanking nucleotide sequence and a second flanking nucleotide sequence substantially homologous with the first flanking sequence. As used herein, the term "substantially homologous with" is used to indicate that the degree of homology is sufficient to result in a desired frequency of recombination. In certain embodiments it may, in order to obtain a desirable maximum frequency of recombination, be advantageous to use direct repeats, i.e. sequences being 100% homologous, whereas in other embodiments where a moderate degree of cell function limitation is desirable, it is appropriate to use repeats which are more or less heterologous, but still allowing a desirable lower frequency of recombination to occur. Accordingly, in the present context, the term "sufficiently homologous" is used to indicate a degree of homology between two flanking nucleotide sequence repeats which results in a desired frequency of recombinational events in a cell containing the gene coding for the toxin polypeptide and a negatively regulatory DNA sequence.

As it also has been mentioned above, the frequency of recombination depends on the lengths of the flanking sequences. In useful embodiments of the invention, flanking sequences are used which have a length being in the range of 100-5,000 base pairs. In certain preferred embodiments, it is advantageous to use flanking sequences, the length of which is in the range of 200-3,000 base pairs. As the flanking sequences can be used any nucleotide repeats of sufficient lengths and homology as it has been defined above. As one useful example of flanking sequences may be mentioned the chloramphenicol resistance gene having a size of about 900 base pairs and which occurs in the plasmid pBR325. Another example of a useful nucleotide sequence which, when inserted as repeats, results in recombination, is a subsequence of the *rrnB* gene isolated from the plasmid pKK3535 (Brosius et al., 1981, Plasmid, 6:112-118) having a size e.g. in the range of 500 to about 3,000 base pairs, such as 598 base pairs.

In one interesting embodiment of the invention, the excisable negatively regulatory DNA sequence operably linked to the gene encoding the cytotoxic polypeptide is a gene encoding an antisense RNA which forms an RNA-RNA duplex with said the messenger RNA of the polypeptide-encoding gene and thereby, when it is expressed, inhibits translation of said gene coding for the polypeptide.

In another useful embodiment of the present invention, the recombinationally excisable negatively regulatory DNA sequence is a gene encoding a polypeptide repressor of transcription of the polypeptide-encoding gene. Such a repressor may, e.g. be a *lac* repressor including the repressor encoded by the *LacIq* gene. It will be appreciated that the negatively regulatory DNA sequence can also be a gene coding for RelB anti-toxin or functionally equivalents hereof.

In a further useful embodiment of the invention, the excisable negatively regulatory DNA sequence is a transcription termination sequence, preventing the transcription of the cytotoxic polypeptide-encoding gene. In one specific embodiment of the invention, such a suitable terminator sequence is the *rpoCt'* transcription terminator isolated from the plasmid pHBA102rpoCt (Squires et al., 1981, Nucleic Acid Res., **9**:6827-6839).

Negatively regulatory DNA sequences which, in accordance with the invention, are suitable, can be isolated from DNA sequences derived from a virus, or a prokaryotic or eucaryotic cell. Thus, sources of the DNA sequence include bacterial chromosomes, bacterial plasmids, prokaryotic viruses, eucaryotic viruses, eucaryotic plasmids, or eucaryotic chromosomes.

In preferred embodiments of the invention, the excisable negatively regulatory DNA sequence and the first and second flanking sequences, both as defined above, is provided in the form of a "cassette" which term is used herein to describe a readily insertable DNA sequence comprising at least the above-mentioned sequences and optionally the gene coding for the cytotoxically active polypeptide, and optionally further nucleotide sequences including as examples a suitable marker such as a gene coding for antibiotic resistance. In the present context, the term "insertable" denotes that the cassette as defined herein is provided with suitable restriction sites at both ends allowing for insertion in a replicon having the same restriction sites. Accordingly, such preferred restriction sites include sites which occur frequently in replicons where insertion is desirable or alternatively, restriction sites which may be easily provided in such replicons.

It will be understood that, in accordance with the invention, a cassette as defined above and which does not comprise the gene coding for toxin polypeptide and opera-

bly linked to the negatively regulatory DNA sequence, may be inserted in a replicon which is different from the replicon containing said gene. Optionally, the cassette as defined above is inserted in a first replicon such as e.g. a transposon and subsequently inserted via the transposon into the chromosome to obtain a cell as defined herein.

As it has been explained above, the activation of certain invertible promoters such as the *fimA* promoter or functional homologues hereof is regulated by the gene products of an *on* gene and an *off* gene. It will be understood that this mechanism of promoter regulation provides the possibility of using the *off* gene or a functional homologue hereof as a negatively regulatory DNA sequence which may be inserted in the microbial cell as defined herein, as a recombinationally excisable DNA sequence in the manner explained in details above. Accordingly, in one embodiment, the present invention provides a microbial cell wherein the toxin-encoding gene is stochastically expressed as a result of recombinational inversion of an invertible promoter sequence.

In plasmids, inherent mechanisms occur whereby multimer resolution of the plasmid during replication takes place. As exemplified by the broad host range plasmid RP4, this resolution system may comprise (1) a gene coding for a multimer resolving enzyme, a resolvase and (2) a site for the site-specific resolvase-mediated resolution. In plasmid RP4 the gene coding for the resolvase is *parA* and the site for the resolution is designated mrs. If two mrs sites are placed in direct orientation, a DNA sequence inserted between those two sites may, if the *parA* gene is present in the same host cell, be deleted at a relatively high frequency whereby a site-specific recombination system is provided. In useful embodiments the *parA* gene may be located in trans.

It has been found that such a site-specific recombination system provides a useful mechanism for stochastically regulating the expression of a gene such as the gene coding for the toxic polypeptide as defined herein, since the site-specific recombination may be used to obtain recombinational excision of a negatively regulatory DNA sequence as defined above.

Accordingly, in one interesting embodiment, the present invention provides a microbial cell as defined herein in which the negatively regulatory DNA sequence is a sequence flanked by a first site for a site-specific resolution recombinase and a second site for

site-specific resolution, the second site being recognizable by the same or a functionally equivalent multimer resolving enzyme as is the first site, whereby the regulatory sequence is recombinationally excisable in the cell. In a specific embodiment, the gene coding for the multimer resolving enzyme is located *in trans* relative to the sites for site-specific resolution. In the present context, one useful example of a suitable gene is the *parA* gene isolated from plasmid RP4.

In accordance with the invention, the method of controlling the survivability of microbial cells can be based on providing in the cells a gene coding for a cytotoxic polypeptide that is structurally and functionally equivalent to the *E. coli* RelE polypeptide (the *relE* gene family). Such a gene can be derived from the chromosome or another replicon of a Gram-negative bacterium including *Enterobacteriaceae* spp. such as *E. coli*, *Hemophilus* spp. such as *H. influenzae*, *Vibrionaceae* spp. such as *V. cholerae*, *Pseudomonadaceae* spp., *Helicobacter* spp. such as *H. pylori* and *Synechosystis* spp, the latter organisms belonging to the group of cyanobacteria. The gene may also be derived from the chromosome and other replicons of Gram-positive bacteria including lactic acid bacteria such as *Streptococcus* spp including *Streptococcus pneumoniae*., *Bacillaceae* spp. such as *B. thuringiensis*, and *Mycobacterium* spp. and from species belonging to *Arhae* such as *Methanococcus jannaschii* and *A. fulgidus*. Such genes include those that are defined herein as belonging to the *relE* gene family. The RelE equivalent polypeptide from *M. jannaschii* was shown to be toxic for *E. coli* when expressed in this organism.

However, genes coding for cytotoxins of other proteic killer systems and which are therefore functional equivalents of the *E. coli* K-12 RelE polypeptide can also be used in accordance with the invention for conditionally controlling the survivabilty of microbial cells. Such genes include the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide and the gene coding for the prophage P1 Doc polypeptide, as described by Jensen et al., 1995.

It will be understood that in this context, the term "functional equivalent" includes variants or derivatives of any of the above first kind of polypeptides the sequences of which have been modified by substitution, deletion or addition of one or more amino

acids and the gene product of which has retained at least part of the function of the gene product of the non-modified sequence.

In accordance with the invention, the *relE* family gene or any gene coding for a toxin of a proteic killer system is provided in the microbial cells at a location where it can be expressed effectively. Thus, in useful embodiments the gene is present on the chromosome of the cells whereas in other embodiments it is preferably located on an extrachromosomal element such as a plasmid or a cosmid. In a specific embodiment, the microbial cells according to the invention do not contain a gene coding for a second type of polypeptide that is capable of counteracting the cell toxic effect of the RelE polypeptide or the functional equivalent hereof.

However, in other useful embodiments, the microbial cells comprise a gene coding for a second kind of polypeptide that is capable of binding to the *relE* polypeptide or the functional equivalent hereof, the binding resulting in that the toxic effect of the RelE polypeptide or the functional equivalent is at least partially counteracted. Such a counteracting second kind of polypeptide is, as it is mentioned above, also referred to herein as an antitoxin or an antidote for the cytotoxic polypeptide.

Although, in certain uses of the present method, it is preferred that the genes coding for both the toxic polypeptide and the antitoxin herefor is under the control of the same regulatory sequences, it may, in other uses, be advantageous that the gene coding for the second kind of polypeptide is operably linked to a different regulatable regulatory DNA sequence as defined above, permitting that the gene coding for the second kind of polypeptide is suppressed under conditions where the gene coding for the RelE polypeptide or the functional equivalent is expressed.

It will be appreciated that the genes coding for the toxin polypeptide and the antitoxin polypeptide, respectively can be present on the same replicon such as a plasmid or on the chromosome, or they can be present on different replicons in the microbial cells.

A useful second kind of polypeptide is the *RelB* polypeptide derived from *E. coli* K-12 which i.a. binds effectively to the *E. coli*-derived RelE polypeptide. However, the regulation of the toxic effect of the first kind of polypeptide can also be based on pro-

viding in the cells a gene coding for a second kind of antitoxically active polypeptide that is functionally equivalent to the *E. coli* RelB polypeptide. Such a gene can be derived from the chromosome or another replicon of a Gram-negative bacterium including *Enterobacteriaceae* spp. such as *E. coli*, *Hemophilus* spp. such as *H. influenzae*, *Vibrionaceae* spp. such as *V. cholerae*, *Pseudomonadaceae* spp., *Helicobacter* spp. such as *H. pylori*, and *Synechosystis* spp belonging to the group of cyanobacteria. Additionally, genes coding for structural and functional equivalents of the *E. coli* RelB polypeptide can be isolated from Gram-positive bacteria including lactic acid bacterial species such as *Streptococcus* spp., *Bacillaceae* spp. such as *B. thuringiensis*, and *Mycobacterium* spp. and from species belonging to *Arhae* such as *M. jannaschii* and *A. fulgidus*. Sequences for the *E. coli* RelB polypeptide and for equivalents isolated from the above organisms are listed in Table 1.6.

Genes coding for functional equivalents of the *E. coli* K-12 RelB polypeptide which in accordance with the invention can be used for containing microbial cells and replicons include the genes coding for the plasmid F CcdA polypeptide, the plasmid R1 Peml polypeptide, the plasmid RP4 ParD polypeptide and the prophage P1 Phd polypeptide.

It will be understood that in this context the term "functional equivalent" includes variants or derivatives of any of the above second kind of polypeptides, the sequences of which have been modified by substitution, deletion or addition of one or more amino acids and the gene product of which has retained at least part of the function of the gene product of the non-modified sequence.

It is a significant objective of the present invention to provide the means of conditionally controlling the survivability of microbial cells that expresses one or more genes coding for a gene product of interest. In accordance with the invention such an objective is pursued for any type of gene products including enzymes such as proteases, enzymes which are effective in degrading carbohydrates such as starch degrading enzymes, lipid degrading enzymes and nucleases.

However, it is of particular interest to provide containment of microbial cells wherein the gene product of interest is selected from an immunologically active gene product,

a gene product that is effective in degradation of an environmental pollutant and a pesticidally active product.

Accordingly, in such specific embodiments the microbial cells are cells which further comprise a DNA sequence that is selected from a sequence coding for an immunologically active gene product, a sequence coding for a pesticidally active gene product and a sequence coding for a pollutant degrading gene product.

In the present context, the term "immunologically active gene product" is used to describe an epitope (antigenic determinant) from a pathogenic organism which, when it is administered to the body of a human or an animal, is capable of stimulating the formation of antibodies therein. A microbial cell as defined herein which contains one or more genes encoding such a gene product can be utilized in the preparation of live vaccines. In the immunization against several pathogens it is considered advantageous to administer live vaccines as compared to killed organisms or antigenic fragments of the pathogen, since the level of immunity conferred by a live vaccine is frequently higher than that conferred by vaccines comprising killed pathogenic organisms or fragments thereof. Most currently used vaccines comprising viable epitope-containing organisms are either based on recombinant non-pathogenic organisms encoding the epitope or they are based on attenuated pathogenic organisms. The cell advantageously contains a multiplicity of genes each of which codes for a specific immunologically active gene product.

However, up till now the use of live vaccines has been limited since it is difficult to obtain the right combination of attenuation, viability and adequate immune response. Furthermore, the deliberate release of genetically engineered microorganisms to the body and to the external environment which is a result of the use of viable recombinant organisms as vaccines, is currently not allowed in any country for reasons of public concern as to the possible long-term environmental impact, in particular the risk of permanent establishment of the GEMs in the environment.

The present invention provides advantageous means of circumventing these problems associated with the use of known GEM-based live vaccines by introducing into a viable epitope-containing cell the regulatably expressible gene coding for a cell toxic

polypeptide as defined above. In particularly interesting embodiments, the invention provides, as a useful basis for a viable vaccine, the microbial cells as defined above whose expression is stochastically induced.

In useful embodiments of the invention, the cell which contains the DNA sequence coding for an immunologically active gene product further comprises means for transporting the epitope, when expressed, to the outer surface of the cell, i.e. translocating it across the cell membrane. Preferably such a translocation is obtained by inserting the gene coding for the epitope into a nucleotide sequence coding for an outer cell surface polypeptide structure such as fimbriae which contains the fimbrillin protein, pili, flagellae or certain other surface proteins including as an example the OM protein found in *Streptococcus* species. By providing the cell with such a hybrid nucleotide sequence being expressible in the cell, the gene product hereof will be a fusion or hybrid protein comprising the epitope and the relevant cell surface structure.

A cell in which a fusion protein is expressed which comprises the epitope fused to a surface structure protein by which the cell can adhere to the mucosal cells of a body to which the cell is administered is considered to be particularly useful in that the epitope will become in close contact with the mucosa and thereby effectively stimulate a protective immune response in the form of the excretion of secretory antibodies of the IgA and IgG classes.

Furthermore, the adhesion of the epitope-carrying cell will ensure that the cell is retained in the human or animal body for a period of time which is sufficient to obtain the desired immune response. It is considered that a satisfactory immunization typically may be obtained if the cell is present in sufficient numbers in a particular body environment such as the intestinal tract for a period in the range of 15-30 days, depending on the nature and the activity of the epitope expressed from the cell.

As it will be understood from the above description of the gene coding for the cell function-limiting toxic polypeptide and the DNA sequence regulating its expression, the present invention may provide useful means of providing live vaccines based on recombinant organisms which are immunologically effective and which can be used

without the risk of undesired spreading of recombinant genes to the microflora of humans and animals or to the outer environment.

In accordance with the invention, a useful cell for the preparation of a live vaccine is one selected from a bacterial species which inherently contains an outer surface structure as mentioned above. Such species include as examples species of *Enterobacteriaceae* such as *Salmonella* and *E. coli* species, *Vibrionaceae* and *Pseudomonadaceae*. It will be understood that strains of such species which are particularly useful in the present invention as the basis of a live vaccine as defined above, are non-pathogenic strains or strains having a low pathogenicity.

The epitope expressed by a cell as defined above may be an epitope derived from any pathogenic organism or agent the obtainment of immunity against which is desirably. Such pathogens include viruses, bacteria and eukaryotic organisms such as fungi, yeast or protozoa.

In commercially important embodiments, the microbial cell comprising the gene coding for a cytotoxic polypeptide contains a nucleotide sequence coding for a pesticidally active gene product. In this context, the term "pesticidally active gene product" is used to denote a product which, when expressed in a cell being released to an environment where there is a need to reduce or eliminate the presence of pests that feed on plants, including insect pests, nematodes and vermins such as rodents or birds, is effective in respect of such pest control.

Such pests are currently controlled by the administration of toxic chemical pesticides to the infestated environment, but recently various naturally occurring pesticidally active organisms including viruses, bacteria and fungi have been used as biological pest control products.

Prominent examples of such pesticidally active organisms include biotypes or strains of the species *Bacillus thuringiensis* that produce crystalline proteins being toxic to insects, in particular to caterpillars, and several viruses being pathogenic for insects in the larval stage or in the adult stage. However, the pesticidal effect of such organisms is frequently less satisfactory and there is a strong need in farming, forestry and horti-

culture to provide improved pesticidally active organisms. One approach to solving this problem is to construct genetically engineered organisms having an increased toxic effect or a better survival rate in the environment. In addition to pesticidally active compounds from *B. thuringiensis*, such compounds are produced by other microbial organisms inlcuding *Bacillus sphaericus*, fungal species, algal species and plants. In accordance with the invention, genes coding for such biopesticides can be inserted and expressed in the biologically contained cells of the invention.

To the extent such improved organisms are developed, their use in the environment will, as a consequence of current public concern of the potential risks involved in deliberate release of such toxic or pathogenic GEMs, only be approved by official environmental agencies if it can be demonstrated that the release does not lead to an undesired propagation or to an extended survival of such organisms in the environment to which they are applied.

The present invention clearly provides the means of limiting the survival in the environment of genetically engineered pesticidally active organisms. As it has been explained above, the rate of expression of the cytotoxic polypeptide can be regulated stochastically and thus the survival rate of pesticidally active cells may conveniently be adapted to any specific need. Also, the cell function-limiting effect of the toxic polypeptide may, in accordance with the present invention, be adjusted by selecting a first kind of polypeptide that has an appropriate cell function-limiting effect.

In another useful embodiment, the invention provides a cell in which the gene coding for a desired gene product is a sequence coding for a pollutant-degrading gene product. It is known that several xenobiotic compounds polluting the outer environment including soil and water can be degraded by microorganisms having an inherent capability of degrading these compounds. Obviously, the technology of genetic engineering provides means of providing improved organisms having an increased pollutant-degrading capacity or having the capacity to degrade a broad range of compounds, in particular hydrocarbons.

However, the public concern as mentioned above are also relevant in this context and accordingly, the present invention provides useful means of providing improved pollu-

tant-degrading microbial cells, the survival of which can be controlled by regulating the expression of the first kind of polypeptide as it is defined above. In particularly preferred embodiments, the cell contains a gene coding for a pollutant-degrading gene product, the expression of which is induced by the presence of a pollutant degradable by the cell.

In addition to the above desired gene products, the microbial cells according to the invention can express any desired gene product including pharmaceutically active products such as e.g. hormones, interleukines and antibiotically active peptides.

As mentioned above, the invention provides in a further aspect a method of confining an extrachromosomal replicon to a microbial cell population. Basically, the method comprises the steps of isolating or constructing a microbial cell containing a gene belonging to the *relE* gene family expressing a first kind of polypeptide that is toxic for the cell and introducing into the cell the extrachromosomal replicon to be confined, which replicon contains a gene coding for a second kind of polypeptide acting as an antitoxin for said first kind of polypeptide, and cultivating the cells under conditions where the genes coding for the first and the second kind of polypeptides are expressed, whereby a daughter cell that does not receive a copy of the extrachromosomal replicon is killed by the first kind of polypeptide being expressed in the absence of expression of the second kind of polypeptide.

In preferred embodiments of such a method the cell population consists of cells that comprise a gene coding for a gene product of interest as defined above.

The above method of confining an extrachromosomal replicon is particularly useful when the replicon is a plasmid that naturally occurs in a host cell in a low copy number. Accordingly, the method is useful for confining a plasmid occurring in the microbial cells at a copy number which is in the range of 1-30 including the range of 1-10 such as the range of 1-5.

Microbial cells to which a replicon can be confined in accordance with the invention include Gram-negative bacterial species such as species belonging to *Enterobacteriaceae*, *Hemophilus*, *Vibrionaceae* and *Pseudomonadaceae* and Gram-positive bacterial

species, fungal cells including yeast cells, animal cells including human cells and insect cells, and plant cells.

In a still further aspect, the invention provides a method of post-segregationally stabilizing a plasmid in a microbial host cell population as described above. As it is mentioned above, the method comprises the steps of (i) inserting into the plasmid a gene coding for a first kind of polypeptide as defined herein and a gene coding for a second kind of polypeptide as also defined herein that is capable of being degraded in the host cell at a higher rate than that at which the first kind of polypeptide is degraded, (ii) cultivating the cell population under conditions where the genes coding for the first kind and second kind of polypeptides are expressed, whereby a daughter cell that does not receive at least one copy of the plasmid is killed as a result of the faster degradation of the second kind of polypeptide.

The invention also provides a recombinant microbial cell as defined above, comprising a gene coding for a first kind of polypeptide. Such a cell can be a bacterium of a Gram-negative bacterial species including *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp or it can be of a Gram-positive bacterial species such as a *Bacillus* species or lactic acid bacterial species, a fungal cell including a yeast cell, an animal cell including a human cell and an insect cell, and a plant cell.

As also mentioned above, the invention pertains in another aspect to a method of limiting the survival of a cell population in a first or a second environment, which method comprises as the first step that the cells are transformed with a gene coding for a cytotoxic polypeptide, which gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide, the gene coding for the prophage P1 Doc polypeptide and a gene coding for a functionally equivalent polypeptide for anyone of said polypeptides.

In a specific embodiment of such a method, the survival of the cell population is limited in a first environment in which the gene is expressed whereby the cell population

is contained in said first environment. In another embodiment, the survival of the cell population is not limited when present in a first environment, which first environment could change to a second environment physically and/or chemically distinct from the first environment, in which first environment the gene whose expression results in the formation of a cytotoxically active polypeptide is not expressed, but the survival of which cell population is limited when transferred to a second environment or when present in a physically and/or chemically changed first environment, where the gene is expressed.

In a still further embodiment of the above method, the survival of a cell population is being limited by providing in the cells a gene coding for a cytotoxic polypeptide which is operably linked to a DNA sequence encoding an antitoxin repressor substance which can undergo a decay when said cells are released to the outer environment to an extent whereby the repressor substance is converted to a non-functional form, whereby as a result of said decay, the function of the cells of the population will be gradually limited.

In yet another aspect of the invention, there is provided a method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is naturally transferable to a second kind of cell, which method comprises as the first step providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides.

In one specific embodiment of such a method the gene product which inhibits the expression of the expression of the gene coding for the polypeptide or the cell function-limiting effect of the polypeptide is selected from the *E. coli* relB polypeptide, the plasmid F CcdA polypeptide, the plasmid R1 PemI polypeptide, the plasmid RP4 ParD polypeptide, the prophage P1 Phd polypeptide and a functionally equivalent polypeptide of anyone of such polypeptides.

The invention also provides a method as defined above of stochastically limiting in an environment the survival of a cell population. Such a method is particularly useful in the containment of recombinant cells which are to released to the outer environment or the animal or human body.

The invention will now be described in further details in the following examples and the drawings wherein

Fig. 1 illustrates *relB*$_{K-12}$::*lacZ* and *relE*$_{K-12}$::*lacZ* translational fusions. Shown are relevant parts of the *lacZ* reporter plasmids pKG4001 (carrying a *relB*$_{K-12}$::*lacZ* fusion) and pKG4002 (carrying a *relE*$_{K-12}$::*lacZ* fusion). Numbers to the right in the Figure indicates *lacZ* expression levels in Miller units. The low expression level of *relE*::*lacZ* in pKG4002 is, in part, due to the presence of an intact *relB* gene located on the plasmid. The *relB* gene product represses the *relBE* promoter c. 130-fold;

Fig. 2 illustrates in vitro translation of *relBE*$_{P307}$-carrying plasmids. Lane 1: pBR322; lane 2: pHA402 (pBR322-*relB*$^+$); lane 3: pHA403 (pBR322-*relBE*+); lane 4: pBR322; lane 5: pHA100 (pBR322-E11 contains the P307 *relBE* genes in their natural context); lane 6: pKG325; lane 7: pHA110 (pBR325-*relB*$^+$);

Fig. 3 shows the structure of expression plasmid pNDM220. The plasmid is a mini-R1 vector whose copy number is amplifiable at 42°C due to the insertion of the temperature inducible λ P_R promoter upstream of the replication control region. The plasmid also carries the cI_{857} temperature-sensitive allele of the cI repressor. Genes shown are *copB* (copy number control), *repA* (initiation of replication), *parM* and *parR* (plasmid stability loci), *bla* (β-lactamase) and *lacI*^{q}. The plasmid contains the LacI regulated pA1/O4/O3 promoter upstream of a multiple cloning site that contains unique *BamHI* and *EcoRI* restriction sites. Thus genes inserted downstream of the promoter are inducible with IPTG;

Fig. 4 illustrates cell killing by *relE*$_{K-12}$ and anti-killing by *relB*$_{K-12}$. Shown are optical density at 450 nm and viable counts as function of time for strains MC1000/pMG223 (*relE*$^+$) (A, B), MC1000/pMG223/pMG2201 (*relB*$^-$ control plasmid) (C, D) and MC1000/pMG223/pMG2202 (*relB*$^+$ plasmid) (E, F). At time zero, transcription of *relE*

on plasmid pMG223 was induced by the addition of IPTG (1 mM). Filled symbols indicate that IPTG was added. As seen from (E) and (F), the presence of *relB* on a second plasmid counteracted *relE* mediated cell killing;

Fig. 5 shows the structure of expression plasmid pBAD33. The plasmid is a medium copy number pACYC-derived vector. The plasmid carries the arabinose inducible pBAD-promoter and the *araC* gene of *E. coli*. Thus upon addition of arabinose to pBAD33 containing cells, genes inserted downstream of pBAD are transcriptionally induced. Genes shown in the Figure are: pACYC-ori: origin of replication; CM(R): gene encoding chloramphenicol acetyl transferase; bla': truncated (nonfunctional) gene encoding β-lactamase; mRNA1 encodes AraC activator protein; pBAD: arabinose-inducible promoter;

Fig. 6 A/B illustrates cell killing by $RelE_{P307}$ and anti-killing by $RelB_{P307}$. Shown are optical density at 450 nm (A,C) and viable counts (B, D) as a function of time for strains MC1000/pHA810/pBR322 (A, B) or MC1000/pHA810/pHA110 (carrying $relB_{P307}$). At time zero, transcription of $relE_{P307}$ on plasmid pHA810 was induced by the addition of arabinose (0.02%). Filled symbols indicate that arabinose was added. As seen from (C) and (D), the presence of $relB_{P307}$ on a second plasmid counteracted $relE_{P307}$ mediated cell killing;

Fig.7 shows maps of pHA705 and pHA715;

Fig. 8 illustrates OD_{450} of MC1000/pHA-Sp2, MC1000/pHA705 and MC1000/pHA715 (+/- IPTG);

Fig. 9 shows viable counts of MC1000/pHA-Sp2, MC1000/pHA705 and MC1000/pHA715 (+/- IPTG);

Fig. 10 is the DNA sequence of the $relBE_{Sp2}$ locus of *S. pneumoniae*;

Fig. 11 is a map of pHA-Sp2;

Fig. 12 is a map of pHAG33;

Fig. 13 is a map of pHAG33-2;

Fig. 14 is a map of pHAG33-3;

Fig. 15 is a map of pHAG33-4;

Fig. 16 illustrates OD_{450} of KT2440/pHAG33-2, KT2440/pHAG33-3 and KT2440/pHAG33-4 (+/- IPTG);

Fig. 17 shows viable counts of KT2440/pHAG33-2, KT2440/pHAG33-3 and KT2440/pHAG33-4 (+/- IPTG);

Fig. 18 is a map of pHA810;

Fig. 19 illustrates Glucose run-out, OD_{450} of MC1000/pHA810;

Fig. 20 illustrates Glucose run-out, viable counts of MC1000/pHA810; and

Fig. 21 illustrates that $RelE_{K12}$, $RelE_{P307}$ and $RelE_{Mj}$ inhibit translation *in vitro*

EXAMPLES

Materials and methods

(i) Bacterial strains

The *E. coli* K-12 strain MC1000 (Casadaban and Cohen, 1980) which contains a chromosomal copy of the *relBE* genes was used as the standard cloning strain and when a chromosomal copy of the *relB* operon was required. The *E. coli* K-12 strain JS115 (*leu, thy, thi, supE, ΔrelB*), which contains a deletion covering the entire *relB* operon was provided by Olle Karlström. The latter strain was used for the regulatory studies of *relBE*.

(ii) Plasmids used

Plasmid pOU253 is a mini-R1 based translational fusion vector carrying the *lacZ* gene of pNM482 (Minton, 1984). The fusion vector is segregationally stable due to the presence of the *parA* system of plasmid R1 (Dam and Gerdes, 1994).

Plasmid pNDM220 is a low copy-number mini-R1 expression vector carrying a multiple cloning site (mcs) placed between the LacI regulated pA1/O4/O3 promoter (Lanzer and Bujard, 1988) and two transcriptional terminators.

pNDM220 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12157.

Plasmid pBD2430 (+388 - +1899) is a pUC18 derivative carrying the complete *relBE* operon and gene *IV* located downstream of *relF* (Olle Karlström, unpublished). The relevant *E. coli* DNA present in pBD2430 is shown in Table 1.1 below.

pBD2430 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12161.

(iii) Plasmids constructed

pKG4001: pBD2430 was digested with *Eco*RI and *Xho*I and the fragment carrying the *relB* promoter (Table 1.1) was inserted into pOU253 producing an in-frame translational fusion between $relB_{K\text{-}12}$ and *lacZ*. Thus, pKG4001 carries a $relB_{K\text{-}12}$::*lacZ* translational fusion.

pOU253 was deposited on 30 April 1998under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12158.

pKG4002: pBD2430 was digested with *Eco*RI and *Bst*1107I and the resulting fragment was inserted into pOU253 producing an in frame translational fusion between $relE_{K-12}$ and *lacZ*. Thus pKG4002 carries an intact $relB_{K-12}$ gene and a $relE_{K-12}$::*lacZ* translational fusion.

pMG223: $relE_{K-12}$ was amplified by PCR on pBD2430 with primers *relE*1B (5'-CCCCGGATCCATAAGGAGTTTTATAAATGGCGTATTTTCTGGATTTTGACG, SEQ ID NO:1) containing the *parA* Shine & Dalgarno (Gerdes and Molin, 1986) and *relE*2 (5'-CCCCCCTCGAGGTCGACTCAGAGAATGCGTTTGACCGC-3', SEQ ID NO:2). The resulting $relE_{K-12}$ carrying fragment was inserted into pNDM220 using the *Bam*HI and *Sal*I restriction sites. Plasmid pMG223 expresses $RelE_{K-12}$ upon addition of IPTG.

pMG2201: this plasmid contains the *Eco*RI-*Eco*47III fragment from pBD2430 inserted between the *Eco*RI and *Sca*I sites of pBR322. Plasmid pMG2201 carries the $relB_{K-12}$ promoter and the 5' part of the $relB_{K-12}$ gene.

pMG2202: pBD2430 was digested with *Eco*RI and *Bst*1107I and the $relB_{K-12}$-carrying fragment was inserted into pBR322 *Eco*RI-*Sca*I. The resulting plasmid carries the *relB* promoter and $relB_{K-12}$.

pHA100: Plasmid pNZ945 is a pBS(+) derivative that carries a 4.3 kb *Eco*RI fragment from plasmid P307. This fragment encodes the RepFIB replicon and the *relBE* genes of P307 (Saul et al., 1989). The 4.3 kb *Eco*RI fragment (designated E11) of pNZ945 was purified and restricted with *Pst*I. The resulting 2.2 kb *Eco*RI-*Pst*I fragment was inserted into pBR322 restricted also with *Eco*RI and *Pst*I. The pBR322-derived plasmid carrying the 2.2 kb *Eco*RI-*Pst*I fragment was designated pHA100. Plasmid pHA100 codes for the entire *relB*E system from P307.

pNZ945 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12160.

pHA110: The 2.2 kb *Eco*RI-*Pst*I fragment of pHA100 was purified and digested with *Apo*I (*Eco*RI isoschizomer). The resulting *Eco*RI-*Apo*I DNA fragment (+1 to +1122)

was inserted into the *Eco*RI site of pKG325 which was constructed as follows: Plasmid pBR325 was restricted with *Pst*I, which has a unique recognition site in the plasmid. The resulting vector DNA fragment was made blunt ended with T4 DNA polymerase according to the manufacturer's instructions, and religated. Transformants that were resistant to chloramphenicol and tetracycline, but sensitive to ampicillin were selected. Thus, pKG325 is a TcR, CmlR and ApS derivative of pBR325.

pKG325 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12159.

Plasmid pHA110 contains the *relB* promoter (p$relB_{P307}$) and gene $relB_{P307}$.

pHA205: Plasmid pHA205 is a derivative of the low copy-number mini-R1 expression vector pNDM220 that contains the *relB* gene from P307. The PCR fragment generated from pNZ945 using primers RelB-P307/1: 5'-CCCCCGGATCCCAGTCTTGAAAGGTGGC-3' (SEQ ID NO: 3) and RelB-P307/2: 5'-CCCCCGAATTCTCATAGGTATTTATCCAG-3' (SEQ ID NO:4) was restricted with *Bam*HI and *Eco*RI and inserted downstream of the pA1/04/03 promoter of pNDM220.

pHA210: Gene $relE_{P307}$ was PCR-amplified from pNZ945 with the primers: *relE*-p307/3 (5'-CCCCGGATCCAGATCTGGATAAATACC, SEQ ID NO:5) and *relE*-P307/2 (5'-CCCCCGAATTCGTAACTTTCTGTGTTTATTGC, SEQ ID NO:6). The resulting PCR DNA fragment was restricted with *Bam*HI and *Eco*RI and inserted into pNDM220 also restricted with *Bam*HI and *Eco*RI. Plasmid pHA210 (+1089 to +1417) is thus a mini-R1 derivative carrying a pA1/04/03::$relE_{P307}$ gene fusion which renders $relE_{P307}$ inducible with IPTG.

pHA215: Genes $relBE_{P307}$ were PCR-amplified from pNZ945 with the primers RelB-P307/1 (5'-CCCCCGGATCCAGTCTTGAAAGGTGGC, SEQ ID NO:3) and *relE*-P307/2 (5'-CCCCCGAATTCGTAACTTTCTGTGTTTATTGC, SEQ ID NO:6). The resulting PCR-generated DNA fragment was restricted with *Bam*HI and *Eco*RI and inserted into pNDM220 also restricted with *Bam*HI and *Eco*RI. Plasmid pHA215 (+840 to +1417) is thus a mini-R1 derivative carrying a pA1/04/03::$relBE_{P307}$ gene fusion rendering the $relBE_{P307}$ genes inducible with IPTG.

pHA402: A *Pst*I-*Aat*II fragment from plasmid pHA205, which carries *lacI*^{q} and the pA1/O4/O3::*relB*$_{P307}$ gene fusion was inserted into pBR322 also restricted with *Pst*I and *Aat*II. Thus, the high copy-number plasmid pHA402 contains a *relB*$_{P307}$ gene which is inducible with IPTG.

pHA403: A *Pst*I-*Aat*II fragment from plasmid pHA215, which carries *lacI*^{q} and the pA1/O4/O3::*relBE*$_{P307}$ gene fusion was inserted into pBR322 also restricted with *Pst*I and *Aat*II. Thus, the high copy-number plasmid pHA403 contains the *relBE*$_{P307}$ genes which can be conditionally induced by the addition of IPTG.

pHA810: A DNA fragment encoding *relE*$_{P307}$ was generated by PCR using primers *relE*-P307/4 (5'-CCCCCGAGCTCAGATCTGGATAAATACC, SEQ ID NO:7) and *relE*-P307/5 (5'-CCCCCGCATGCGTAACTTTCTGTGTTTATTGC, SEQ ID NO:8). The fragment was digested with *Sac*I + *Sph*I and inserted into the expression plasmid pBAD33 also digested with *Sac*I + *Sph*I. The resulting plasmid, pHA810 (+1089 - +1417), contains the pBAD::*relE*$_{P307}$ gene fusion that renders *relE*$_{P307}$ inducible with arabinose.

An overview of the bacterial strains and plasmids used herein is shown in Table 0.1 below.

Table 0.1. Bacterial strains and plasmids

Strains	genotypes			Reference/Source
MC1000	*Δlac leu ara*			Casadaban & Cohen, 1980
JS115	*ΔrelB leu thy thi supE*			J. P. Bouche, unpublished
Plasmids	**Replicon**	**Resistance[a)]**	***relBE* co-ordinates[b)]**	**Reference/Source**
pOU253	mini-R1	Ap^R	none	lab. collection
pBAD33	pACYC	Cml^R	none	Guzman et al., 1995
pNDM220	mini-R1	Ap^R	none	Gotfredsen & Gerdes, 1998
pBR322	ColE1	Ap^R, Tc^R	none	Bolivar et al., 1978
pKG325	pBR325	Tc^R	none	lab. collection
pBD2430	pUC	Ap^R	+388 - +1899	Olle Karlström collection
pNZ945	pUC	Ap^R	+1 - +4298	Saul et al., 1989
pKG4001	mini-R1	Ap^R	+388 - +596	Gotfredsen & Gerdes, 1998
KG4002	mini-R1	Ap^R	+388 - +921	Gotfredsen & Gerdes, 1998
pHA100	pBR322	Tc^R	+1 - +2198	Grønlund & Gerdes, 1998
pHA110	pBR325	Tc^R	+1 - +1122	Grønlund & Gerdes, 1998
pHA205	mini-R1	Ap^R	+840 - +1111	Grønlund & Gerdes, 1998
pHA210	mini-R1	Ap^R	+1089 - +1417	Grønlund & Gerdes, 1998
pHA215	mini-R1	Ap^R	+840 - +1417	Grønlund & Gerdes, 1998
pHA402	pBR322	Tc^R	+840 - +1111	Grønlund & Gerdes, 1998
pHA403	pBR322	Tc^R	+840 - +1417	Grønlund & Gerdes, 1998
pHA810	pACYC	Cml^R	+1089 - +1417	Grønlund & Gerdes, 1998
pMG223	mini-R1	Ap^R	+733 - +1020	Gotfredsen & Gerdes, 1998
pMG2201	pBR322	Tc^R	+388 - +597	Gotfredsen & Gerdes, 1998
pMG2202	pBR322	Tc^R	+388 - +921	Gotfredsen & Gerdes, 1998

a) Tc^R, tetracycline resistance; Ap^R, ampicillin resistance; Cml^R, chloramphenicol resistance.

b) Co-ordinates refer to Table 1.1 ($relBE_{K\text{-}12}$, pMG-plasmids) or Table 1.2 ($relBE_{P307}$, pHA-plasmids)

(iv) Growth media and antibiotics

The growth medium was LB medium (Bertani, 1951) or A + B minimal medium (Clark and Maaløe, 1967) supplemented with 0.2% glucose and 1% casamino acids. For growth on solid media, LA-plates were used. LA is LB containing 15 g agar per litre. All media were supplemented with 50 μg/ml thymine for growth of the strain

JS11507-05-99Δ*relBEF*$_{K-12}$. Antibiotics were added at the following concentrations: ampicillin, 30 μg/ml, and tetracycline, 10 μg/ml. When indicator plates were used X-gal (5-Bromo-4-chloro-3-indolyl-ß-D-galactoside) was added to a final concentration of 40 μg/ml.

(v) Conditions of cell growth.

Cells were diluted in LB + antibiotics from an overnight culture to an OD_{450} of 0.005. The cultures were then grown at 37°C until an OD_{450} of 0.4 and then diluted to an OD_{450} of 0.01 in 37°C LB containing 1 mM IPTG and antibiotics. Samples for OD_{450} measurements and viable counts were taken at the time points indicated. Viable counts were made by plating dilutions of the cultures onto LA plates containing the proper antibiotics.

(vi) Coupled *in vitro* transcription and translation.

The reactions were performed using the *E. coli* S30 Extract System For Circular DNA as described by the supplier (Promega Corp.). 4 μg of DNA was used in all reactions. The reactions were run on a 16% Tricine-SDS-PAGE gel essentially as described by Schäger and von Jagow (1987).

(vii) ß-galactosidase assays.

ß-galactosidase assays were performed essentially as described by Miller (1972).

(viii) Homology search.

BLAST searches were performed at the GENESTREAM BLAST network server CRBM Montpellier, France. Standard conditions were used except that the blosum 80 matrix was used.

EXAMPLE 1

The occurrence of *relBE* operons in bacteria and *Archae*

1.1. Nucleotide sequence of the *relBE* operon of *E. coli* K-12

The DNA sequence of the *relBE* operon from *E. coli* K-12 is shown in Table 1.1. In this Table the transcriptional start site of the *relBE* mRNA is indicated with two asterisks (heterogeneity). IR indicates inverted repeats in the promoter and terminator regions. Start codons and stop codons are shown in bold. The transcriptional termination point (*ttp*) of the *relBE* mRNA is also indicated with a vertical arrow. The DNA sequence is from Bech et al., 1985.

By visual inspection of the $relB_{K\text{-}12}$ and $relE_{K\text{-}12}$ genes there was found striking similarity with the so-called "proteic plasmid stabilization systems" as described by Jensen and Gerdes (1995). First, $relE_{K\text{-}12}$ codes for a very basic protein ($RelE_{K\text{-}12}$; pI = 9.7) of 95 amino acids (aa), and $relB_{K\text{-}12}$ codes for a very acidic protein ($RelB_{K\text{-}12}$; pI = 4.8) of 79 aa.

The sequences of proteins $RelB_{K\text{-}12}$ and $RelE_{K\text{-}12}$ are shown in Tables 1.5 and 1.6, respectively. These Tables show multiple sequence alignments of the RelB and RelE gene families. Conserved amino acids at a given position are shown with shading as follows: two amino acids are considered conserved if they both belong to one of the following groups: group 1: D and N; group 2: E and Q; group 3: S and T; group 4: K and R; group 5: F, Y and W; group 6: L, I, V and M. Light grey shading indicates 60-80% conservation, dark grey indicates 80-99% conservation and black indicates 100% conservation. Note in Table 1.6 the fully conserved glycine at position 69 (G in consensus line) and the fully conserved arginine at position 79 (R in consensus line). The entrez database accession numbers of the protein sequences are given in Tables 1.3 and 1.4.

The $relB_{K\text{-}12}$ and $relE_{K\text{-}12}$ genes are co-transcribed with a third gene, *relF* (also denoted *orf-3* or *hok*C), which is homologous to the *hok* gene from plasmid R1 (Gerdes et al., 1986). The start site (i.e. the 5'-end) of the *relBE* mRNA was determined to be 31 nu-

cleotides upstream of the *relB*$_{K-12}$ AUG start-codon (Bech et al., 1985) and was confirmed (M. Gotfredsen and K. Gerdes, 1998). Inverted arrows in the *relB*E promoter region (Table 1.1) indicate putative binding sites for regulators of transcription (i.e. the RelB$_{K-12}$ and RelE$_{K-12}$ proteins themselves).

The properties described above suggested that RelE could be a cytotoxin and that RelB could be an antitoxin which counteracts the toxicity elicited by RelE.

Table 1.1. DNA sequence of the *relBE* operon from *E. coli* K-12 (SEQ ID NO:9)

```
   1  CTTAATTTCA GGCCCCATCG GATCACACAT GGAGAGTTTT TATGAATAAC
  51  CCCGTCTGTC TTGATGACTG GTTGATTGGC TTTAAAAGCT TGTTGACAGG
 101  GGTAAACGTT CGGCAATAAT TTTCTGCCGC ATGCGGGTGT TGCATAAAAC
 151  GTGTTACGTT CCTTTATCGA CAGGTCAGGT CACCGCTCAC CCGCCGACGA
 201  GAAAGCAACA CTGACATGCT AAAGCAAAAA ATAGATGAAT AAGTTGAGTT

 251  GTGCATATGT AGCCTGACCG TCACAAAGTA TATGGTGTCT GTACCAGTAA

 301  GATGATGGCC GGACTCTTTA AAAACGAGCT GACCTGCACA ATACAGGATG

 351  GACTTAGCAA TGGCTGCTCC TGGCACAAAG CGGACAGTGA TCACCGTTCT

 401  TACGACTACT TTCTGACTTC CTTCGTGACT TGCCCTAAGC ATGTTGTAGT

                    **→relBEF mRNA                    relB start
 451  GCGATACTTG TAATGACATT TGTAATTACA AGAGGTGTAA GACATGGGTA
      ---- ---- --→IR←--- ------ --

 501  GCATTAACCT GCGTATTGAC GATGAACTTA AAGCGCGTTC TTACGCCGCG

 551  CTTGAAAAAA TGGGTGTAAC TCCTTCTGAA GCGCTTCGTC TCATGCTCGA

 601  GTATATCGCT GACAATGAAC GCTTGCCGTT CAAACAGACA CTCCTGAGTG

 651  ATGAAGATGC TGAACTTGTG GAGATAGTGA AAGAACGGCT TCGTAATCCT

                          End relB      Start relE
 701  AAGCCAGTAC GTGTGACGCT GGATGAACTC TGATGGCGTA TTTTCTGGAT

 751  TTTGACGAGC GGGCACTAAA GGAATGGCGA AAGCTGGGCT CGACGGTACG

 801  TGAACAGTTG AAAAAGAAGC TGGTTGAAGT ACTTGAGTCA CCCCGGATTG

 851  AAGCAAACAA GCTCCGTGGT ATGCCTGATT GTTACAAGAT TAAGCTCCGG

 901  TCTTCAGGCT ATCGCCTTGT ATACCAGGTT ATAGACGAGA AAGTTGTCGT

 951  TTTCGTGATT TCTGTTGGGA AAAGAGAACG CTCGGAAGTA TATAGCGAGG

                    End relE
1001  CGGTCAAACG CATTCTCTGA ACCAAAGCAT GACATCTCTG TTTCGCACCG
```

```
                                                        Start hokC (relF)
1051  AAGGTGACAC TTCTGCTTTG CGTTGACAGG AGAAGCAGGC TATGAAGCAG

1101  CAAAAGGCGA TGTTAATCGC CCTGATCGTC ATCTGTTTAA CCGTCATAGT

1151  GACGGCACTG GTAACGAGGA AAGACCTCTG CGAGGTACGA ATCCGAACCG

                                                          End hokC
1201  ACCAGACGGA GGTCGCTGTC TTCACAGCTT ACGAACCTGA GGAGTAAGAG

1251  ACCCGGCGGG GGAGAAATCC CTCGCCACCT CTGATGTGGC AGGCATCCTC

1301  AACGCACCCG CACTTAACCC GCTTCGGCGG GTTTTTGTTT TTATTTTCAA
                        ---- -- IR ---- - ttp

1351  CGCGTTTGAA GTTCTGGACG GTGCCGGAAT AGAATCAAAA ATACTTAAGT
        (data base accesion number  X02405)
```

Table 1.3. *relE* homologues from Gram-positive and Gram-negative bacteria and *Archae*

Bacterial species	entrez accession	gene[a)]	Number of aa	MW (kD)	pI
Gram-negative bacteria:					
E. coli K-12	132284	$relE_{K-12}$	95	11.2	9.7
E. coli K-12	984581	$relE_{SOS}$ [b)]	92	10.8	9.5
E. coli plasmid P307	516611	$relE_{P307}$	95	11.2	9.9
H. influenzae	1175293	$relE_{Hi}$	102	11.9	6.7
V. cholera	396846	$relE_{Vc}$	96	11.2	9.9
H. pylori	2314031	$relE_{Hp}$	88	10.4	7.9
Synechosystis	1653777	$relE_{Sy}$	120	13.7	7.9
Gram-positive bacteria:					
B. thuringiensis	520407	$relE_{Bt}$	74	8.6	9.7
M. tuberculosis#1	2612811	$relE_{Mt1}$	87	10.2	11.0
M. tuberculosis#2	2695832	$relE_{Mt2}$	97	11.1	9.5
Archae:					
M. jannaschii#1	1498833	$relE_{Mj1}$	90	11.0	10.2
M. jannaschii#2	1499953	$relE_{Mj2}$(*)	88	10.6	10.0
M. jannaschii#3	1591583	$relE_{Mj3}$(*)	91	11.1	10.1
A. fulgidus#1	2648176	$relE_{Af1}$	87	10.6	10.3
A. fulgidus#2	2649499	$relE_{Af2}$	92	11.0	9.9
A. fulgidus#3	2649496	$relE_{Af3}$	85	10.0	10.0
A. fulgidus#4	2649514	$relE_{Af4}$	86	10.2	9.9

a) *relE* homologues marked with (*) are not located adjacent to a *relB* partner

b) The $relBE_{SOS}$ system of *E. coli* K-12 contains a LexA binding-site in the promoter region (Lewis et al., 1994)

Table 1.4. *relB* homologues from Gram-positive and Gram-negative bacteria and *Archae*

Bacterial species	entrez accession	gene[a)]	Number of aa	MW (kD)	pI
Gram-negative bacteria:					
E. coli K-12	132283	$relB_{K-12}$	79	9.1	4.8
E. coli K-12	984582	$relB_{SOS}$ [b)]	86	9.4	5.2
E. coli K-12	984588	$relB_{K-12,2}$(*)	97	11.2	5.5
E. coli plasmid P307	516610	$relB_{P307}$	83	9.2	4.4
S. typhimurium	731639	$relB_{St}$(*)	68	7.6	5.3
H. influenzae	1573712	$relB_{Hi}$	98	11.0	4.7
V. cholera	396847	$relB_{Vc}$	82	8.9	4.4
H. pylori	2314037	$relB_{Hp}$	95	11.4	9.8
Synechosystis	1653776	$relB_{Sy}$	86	9.9	4.7
Gram-positive bacteria:					
B. thuringiensis	520406	$relB_{Bt}$	85	10.1	4.5
M. tuberculosis#1	2612810	$relB_{Mt1}$	93	10.2	4.6
M. tuberculosis#2	2695833	$relB_{Mt2}$	89	9.8	5.1
Archae:					
M. jannaschii#1	1498832	$relB_{Mj1}$	82	9.6	4.5
A. fulgidus#1	2648190	$relB_{Af1}$	65	7.8	4.8
A. fulgidus#2	2649516	$relB_{Af2}$	62	7.4	4.3
A. fulgidus#3	2649510	$relB_{Af3}$	72	8.5	4.5
A. fulgidus#4	269513	$relB_{Af4}$	57	6.7	4.1

a) *relB* homologues marked with (*) are not located adjacent to a *relE* partner.
b) The $relBE_{SOS}$ system of *E. coli* K-12 contains a LexA binding-site in the promoter region (Lewis et al., 1994).

Table 1.5. Alignment of *relE* homologues from Gram-positive and Gram-negative bacteria and ***Archae*** (SEQ ID NOS:10-26)

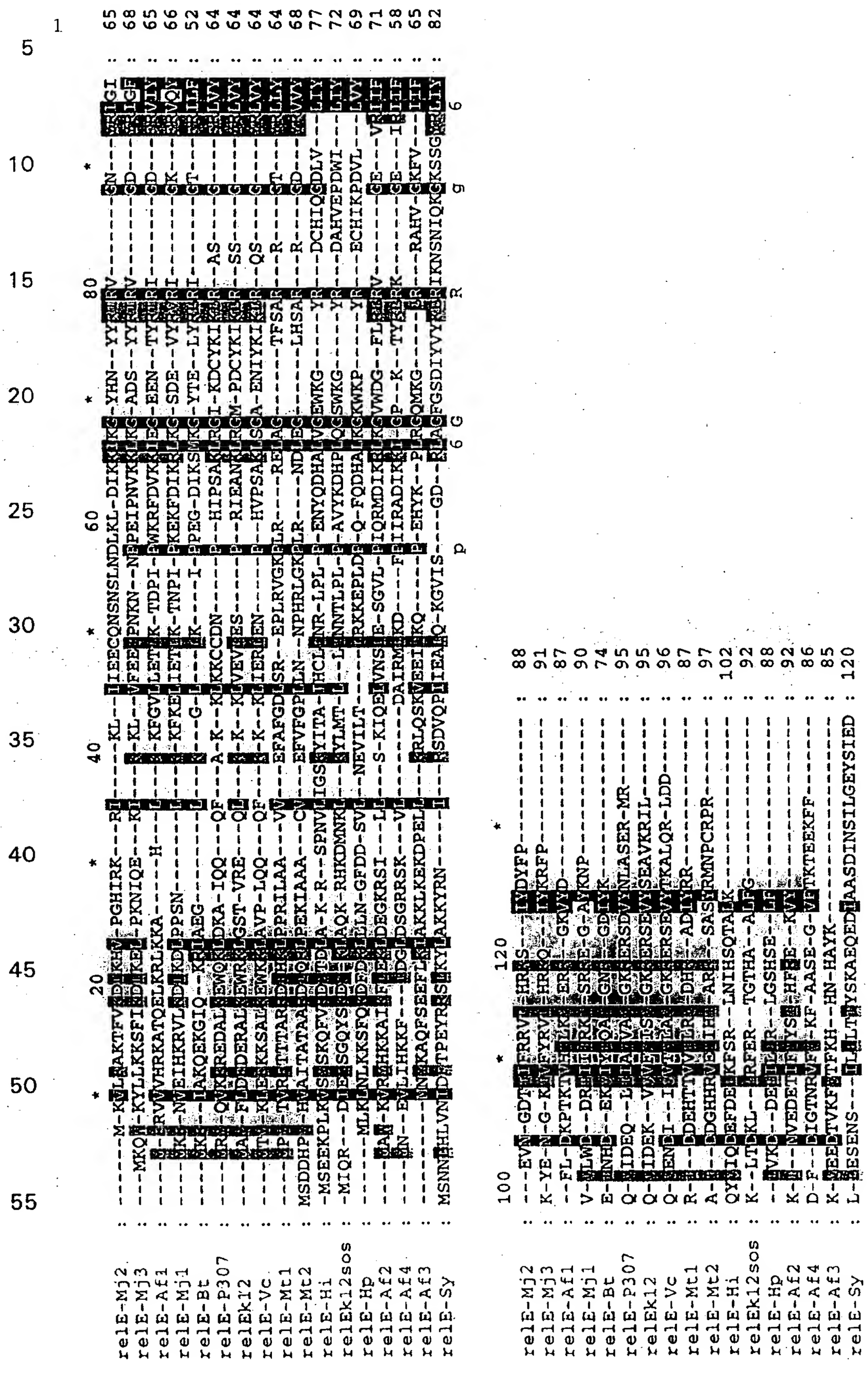
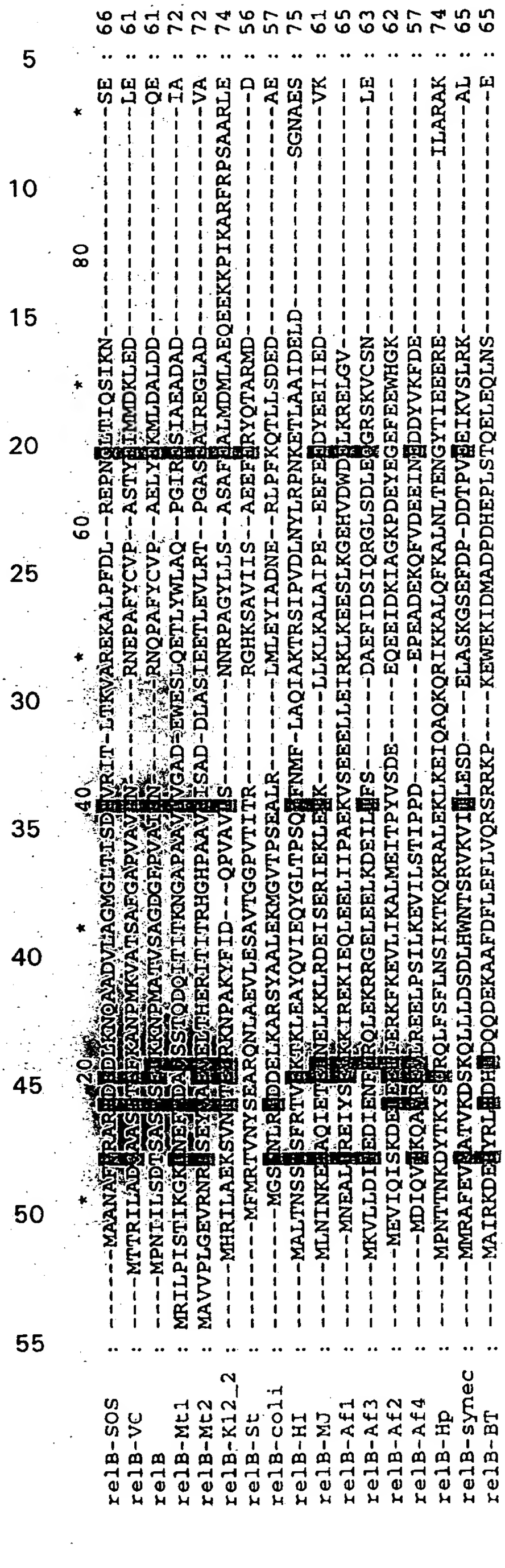


Table 1.6. Alignment of *relB* homologues from Gram-positive and Gram-negative bacteria and *Archae* (SEQ ID NOS:27-43)



1.2. Nucleotide sequence of the *relBE* operon of plasmid P307

By database searching it was found that the *E. coli* plasmid P307 codes for a gene system which exhibits both structural and sequence similarity with the *E. coli relBE* genes described above.

The DNA sequence of the $relBE_{P307}$ genes is shown in Table 1.2. The transcriptional start site of the *relBE* mRNA is indicated with an asterisk, and the -10 and -35 sequence elements of the *relBE* promoter are underlined. The Shine & Dalgarno sequence of the *relB* and *relE* genes are doubly underlined. The DNA sequence is from Saul et al., 1989.

Again, $relE_{P307}$ codes for a very basic protein of 95 aa (pI = 9.9), and $relB_{P307}$ codes for a very acidic protein of 83 aa (pI = 4.4), see Tables 1.3 and 1.4. The protein sequences of $RelE_{P307}$ and $RelB_{P307}$ are also shown in Tables 1.5 and 1.6, respectively. The start site (i.e. the 5'-end) of the $relBE_{P307}$ mRNA was determined to be located 27 nucleotides upstream of the $relB_{P307}$ AUG start codon. Inverted arrows in the $relBE_{P307}$ promoter region (Table 1.2) indicate putative binding sites for regulators of transcription (i.e. the $RelB_{P307}$ and $RelE_{P307}$ proteins).

Table 1.2. DNA sequence of the *relBE* operon from the *E. coli* plasmid P307 (SEQ ID NO:44)

```
      301  GAGTATCATA TTAGGATACG GGTGGGTGAC GCCCACCTCT GGCATAGAAC

      351  GGACATTCAT TGATGCCATG CCAGAATGGA CGTTCAGGTT ATTCCGTCCA

      401  GTTCTGCTGG CAACGCGAGA TCTCCCCTGG TATAGTGATG CCACAGCAAA

      451  GCGCTCAAAC AGGGATAATA TGATGGAAAT CAAGGCTCAA CAGTTTTGTC

      501  ACATCAACGG GGCGGCAAGT CCTTACTGAC AACGGACAAC AAGGTATGGG

      551  CGGCGTGGCG GGTATCGGTT CCACGACTGA AAAGCATCAG GGGCGCGTGG

      601  CGGAAGCGAT TTTTGCGAAC TGCGCGGAAC TGGATAACGA CCAGCTTAAC

      651  GAGATCATCG AGTGGGTTCG GCTCTATCAG CGCTGAATGC CACTATCAGG

      701  CTGCGCAAGC GGCCTTTTTT ACGCCCCTTG TTTAATTCCC GCACTACCTG

                                                              -35
      751  GACGTTCAGG TGATTCTGTC CATCTGTACA AAAAACAATA AAAGACTTGT

                                -10           *→ relBE(P307) mRNA
      801  TAACAGGTCA TGTAAGGAGT ATCTTTGAGA CTGGTTAAAC AGTCTTGAAA

           SD       start relB
      851  GGTGGCCTAT GCCTAACATT ATTCTCAGTG ATACAAGCGC CAGTGTCAGC

      901  GAGCTGAAGA AAAACCCGAT GGCGACAGTC AGCGCCGGTG ATGGTTTCCC

      951  GGTCGCTATC CTGAACCGTA ATCAGCCTGC TTTCTACTGT GTACCCGCAG

     1001  AGCTGTACGA AAAGATGCTT GATGCCCTAG ACGATCAGGA GTTGGTTAAA

                                                              SD
     1051  CTGGTAGCCG AACGCAGCAA CCAACCGCTG CATGATGTAG ATCTGGATAA

           end relB/start relE
     1101  ATACCTATGA GGTATCAGGT AAAATTCAGG GAAGATGCGC TGAAAGAGTG

     1151  GCAAAAACTG GACAAGGCTA TTCAGCAACA GTTTGCGAAA AAGCTAAAAA

     1201  AGTGCTGTGA CAATCCGCAT ATTCCTTCCG CAAAACTGCG TGGGATAAAG

     1251  GACTGCTACA AAATAAAATT ACGTGCGTCA GGTTTTCGCC TGGTCTATCA

     1301  GGTGATTGAC GAACAATTAA TTATCGCTGT TGTAGCTGTG GGTAAACGTG

                                                        end relE
     1351  AGCGCAGTGA CGTTTATAAT CTTGCCAGCG AAAGAATGAG ATAAAAGCAA
```

```
1401  TAAACACAGA AAGTTACTCT GGCGTTATGG GGTAATGCAA AGTATGAGTC

1451  GTAGAGGGAA TTGCCTGGAT AATTCGCCGA TGGAAAGAGT CTTTCGCAGC

1501  CTTAAAAGTG AATGGCTTCC GAAAGGTGGT TATGGTGATT TTAGCCATGC
      (database accession number M26308)
```

1.3: Nucleotide sequence and proteins of a *relBE* homologous operon from *Bacillus thuringiensis*

Using BLAST database searching (Altschul et al., 1990) it was found that transposon Tn5401 from the Gram-positive organism *B. thuringiensis* contains, in one end or asymmetrically located, a two-component system which exhibits both structural and sequence similarity with the above described *relBE* systems from *E. coli*. This homology is surprising given that it has not previously been described that *relBE*-like genes are found in organisms other than *E. coli*.

The nucleotide sequence of the *relBE* operon from Tn5401 is shown in Table 1.7. In this Table the transcriptional start-site of the *relBE* mRNA is indicated with an asterisk (Baum, 1994). IR indicates inverted repeats in the $relBE_{Bt}$ promoter region. Start codons and stop codons are shown in bold. The Shine & Dalgarno sequence of the $relB_{Bt}$ gene is doubly underlined. The DNA sequence is from Baum et al., 1989.

The $relE_{Bt}$ gene codes for a very basic protein of 74 aa (pI = 10.6) and the $relB_{Bt}$ gene codes for an acidic protein of 87 aa (pI = 4.4). The protein sequences of $RelE_{Bt}$ and $RelB_{Bt}$ are aligned with the other RelE and RelB homologues in Tables 1.5 and 1.6, respectively. The modular, structural and physico-chemical similarities between the *B. thuringiensis* system and the *E. coli* systems suggested that the genes may exert similar functions in very different bacteria.

Table 1.7: DNA sequence of the *relBE* operon from the Gram-positive organism *B. thuringiensis* (SEQ ID NO:45)

```
3701  CTCGTTTTTT CTGTTGGTAC AAACTTAATT GATTTTGAAT AATTTGTTTG

3751  TACCAGTCCT TTTTGCTTAG CCCAGTCAAA ATAACGTTTG ATTGAATTAA

3801  TGCGCCGGTT AATCGTAGAA GGTTTTAGTA ATCTTGTAAC TTGCATATGC

3851  CCTCGATATC GAGCAATAGT GCGAGCGGTA ACTTCTATTG GATGAAAAAG

3901  AGTATCCTCA GCATGTTTTC CCCACACATT TTCAAACCAA AATACAAAAT

3951  CTTTTAAATC ACTCGTATAT TCTTTTAGTG TTTTTGTATG CAAATCTCCT

                                                      -35
4001  TCTTGAGATA AGCTAGAAAT AAAATCGGAA ATCAAAGATG TTGCTTGTAT

4051  AGAAATTGTT TTAGTGGAAT GCATAAATAC CTCCTCTTTT ATTGACTTAC

                  -10          *→ relBE_Bt mRNA
4101  ATTAGCGGAC ATGATATTTT AATCTTATCA ATTATGTTAG CGGACATCAA

4151  ACATTTATTT TCCCACACTT CATGTCCACT AATATTAATT AGTGGACATT
                              --------- --→ IR ←-- ---------
                        SD              Start relB
4201  TAAAACTATC TCGAAAGTAG GTGTAACACA TGGCTATTCG TAAAGATGAA

4251  TTGTATCGGT TAATTGATCA CCTGGATCAA CAAGATGAAA AAGCAGCATT

4301  TGACTTTTTA GAATTTCTTG TTCAACGGTC AAGAAGAAAA CCTAAAGAAT

4351  GGGAAAAAAT TGATATGGCA GATCCTGATC ATGAACCGCT GTCTACACAA

4401  GAGTTAGAAC AGTTAAACAG TGAAGAAGGA TATGTATCAG GGGAGGACGC

                                        End relB    Start relE
4451  AAAACGTGAA TTCGGACTAC AAATTGATTT ACCATAAGTC CGCGGTGAAA

4501  TTTATTGCAA AGCAAGAAAA AGGGATTCAA AAAAGAATTG CAGAAGGATT

4551  GAAGGGACTT CTTAAGATTC CTCCTGAAGG AGATATTAAA AGTATGAAAG

4601  GTTACACAGA ACTATATCGA TTACGGATTG GAACCTTTCG AATTTTATTT
```

4651 GAAATAAATC ATGATGAGAA AGTCATATAC ATACAAGCAA TTGGAAATCG

End *relE*

4701 TGGTGACATC TATAAA**TAA**G GCAAACATGC ATTTTTAAAA GAAAGGTCTT

4751 CTGAATCGAA GAACCTTCCT TTTTTGTGTG CGAATAATGT CCGCTAATGC

4801 TTGTTGCGTG ATTCTGTTCC ATTGCTACAC ATACCCC

(database accession number U03554)

1.4. The archaeon *Methanococcus jannaschii* encodes a *relBE* homologous system

Again using database searching it was found that the completely sequenced genome of the methanogenic archaeon *Methanococcus jannaschii* codes for three *relE* homologous genes, one of which are located just downstream of *relB* homologous genes. This finding was surprising since, in many respects, archaeal organisms are more similar to eukaryotes than to bacteria (e.g. in their macromolecular synthesis apparatuses).

The DNA sequence of the $relBE_{Mj1}$ system is shown in Table 1.8. In this Table start codons and stop codons are shown in bold. The DNA sequence is from Bult et al., 1996.

Gene $relE_{Mj1}$ codes for a very basic polypeptide of 90 aa (pI = 11.0) and gene $relB_{Mj1}$ codes for an acidic polypeptide of 82 aa (pI = 4.4). The aa sequences of the $RelE_{Mj1}$ and $RelB_{Mj1}$ proteins are aligned with the other RelBE homologues in Tables 1.5 and 1.6, respectively. Thus, these basic similarities suggested that the $relBE_{Mj1}$ system may carry out similar or related functions in bacteria and archae. The properties of the second and third *relE* homologues of *M. jannaschii* are also given in Table 1.3. These comparisons show that *M. jannaschii* codes for one complete *relBE* homologous gene system and for two *relE* homologues without an adjacent *relB* partner.

Table 1.8. DNA sequence of a *relBE* homologous gene system from the archaeon *Methanococcus jannaschii* (SEQ ID NO:46)

751 CCGATACCGT TGCTGGAGAC ATAGCTGGAG CTTTGAAGGC GGAGAAGCTT

801 ATTTTAATAA CAGATGTTGA TGGAATAATG GATGATATAA ATAATCCAGA

851 GACGTTGCAT AGAAAATTAA CAGCTTCAGA ACTAAAAGAA ATGATAGAAG

901 ATGGAAGAAT AAAGGGAGGG ATGATTCCAA AGGCTGAAAG TGCCTTATAT

951 GCCTTAGAGC ATGGAGTTAA GAGCGTTCAT ATAATAAATG GAAAGATTCC

1001 TCATGCTTTG TTGTTGGAGA TATTTACAGA GGAGGGTATT GGGACGATGA

1051 TAACAAGAGA TTAAAGTTTT TATATTATAA ACTACTTAAG AATTAAAATA

Start $relB_{Mj1}$

1101 AGACAAATAA GGGGATAACT **ATG**CTCAATA TAAACAAAGA GATAGCACAA

1151 ATAGAAACTG AATTGAATGA ATTGAAAAAA TTGAGAGATG AAATCTCTGA

1201 AAGGATTGAA AAATTAGAAA TAAAGTTATT AAAATTGAAA GCATTAGCTA

1251 TTCCAGAGGA GGAATTTGAA GAGGATTATG AAGAAATTAT AGAAGATGTT

1301 AAAAAATCTC TGGATAAAAA AGAGACTGTG CCAGCAGAAG AGGCTTTGAA

End $relB_{Mj1}$/start $relE_{Mj1}$

1351 AGAATTGGGA TTATT**ATGA**A GTTTAACGTT GAGATACATA AAAGAGTCTT

1401 AAAAGATTTA AAGGATTTGC CTCCCTCAAA CTTAAAGAAG TTTAAAGAAC

1451 TAATAGAAAC ATTAAAAACC AATCCCATTC CAAAAGAAAA ATTTGATATT

1501 AAAAGATTAA AAGGCAGTGA TGAGGTTTAT AGAGTTAGAA TTGGAAAATT

1551 TAGAGTTCAA TATGTTGTTT TATGGGATGA TAGAATAATA ATAATTAGAA

End $relE_{Mj1}$

1601 AGATAAGTAG AAGAGAAGGA GCTTATAAAA ATCCC**TAA**GC TATTAAAAAT

1651 TCTAATGGCT ACATTTTTAT ATCTCTTTTC TTAATTCAAA TAGAAAAAAC

1701 AGATTCGGCT GATACCATGA TTATTCTTTT AGATTTAAAT GGAACAATAG

(database accession number U67464)

1.5. *relBE* homologous genes are ubiquitous in prokaryotes

Further *relBE* homologous two-component systems were discovered. The corresponding RelB and RelE homologous proteins are aligned in Tables 1.5 and 1.6, respectively. It appears that *relE* homologous genes are present in a wide variety of Gram-negative bacteria (*E. coli*, *H. influenzae*, *V. cholera*, *H. pylori* and *Synechosystis*), in Gram-positive bacteria (*B. thuringiensis* and *M. tuberculosis*) and in *Archae* (*M. jannaschii* and *A. fulgidus*). Most strikingly, the archaeon *A. fulgidus* contains four complete *relBE* homologous gene systems.

A number of features become evident from the alignments of the proteins (Tables 1.5 and 1.6) and from the properties listed in Tables 1.3 and 1.4. First, all RelE homologues are basic with pH's around 8-10 whereas the *RelB* homologues are acidic with pI's about 4-5. Secondly, the RelE proteins are in general slightly larger (90-120 aa) than the *RelB* homologues (70 - 80 aa). Thirdly, the start codons of the *relE* genes are juxtaposed or even overlap with the stop codons of the linked *relB* partner, thus indicating translational coupling of *relE* to *relB*. These properties suggest that the proteins could exert similar functions in very different organisms.

EXAMPLE 2

Demonstration of translation of the *relB*$_{K-12}$ and *relE*$_{K-12}$ genes

Using the low copy-number *lacZ* fusion vector pOU253 (Table 0.1) in frame gene fusions between *relB*$_{K-12}$ and *relE*$_{K-12}$ and the *lacZ* gene were constructed (see Materials and methods). Thus plasmid pKG4001 (+388 to +596) carries a fusion between *relB*$_{K-12}$ and *lacZ*, and pKG4002 (+388 to +921) carries a fusion between *relE*$_{K-12}$ and *lacZ*. The structure of the relevant parts of these reporter plasmids are shown in Fig. 1. When present in strain MC1000, both plasmids expressed significant amounts of β-galactosidase fusion proteins, indicating that genes *relB*$_{K-12}$ and *relE*$_{K-12}$ are translated (Fig. 1). The *relE*$_{K-12}$-*lacZ* fusion (pKG4002) expressed significantly lower amounts of β-galactosidase than the *relB*$_{K-12}$-*lacZ* fusion, mainly because pKG4002 encodes an intact *relB*$_{K-12}$ gene which produces the RelB$_{K-12}$ autorepressor which inhibits transcription from the *relB* promoter.

EXAMPLE 3

Demonstration of translation of the *relB*$_{P307}$ and *relE*$_{P307}$ genes

To detect authentic RelB$_{P307}$ and RelE$_{P307}$ proteins, in vitro translation reactions were carried out using high copy number pUC-plasmids carrying genes *relE*$_{P307}$ (pHA403), *relB*$_{P307}$ (pHA402) or both genes (pHA100) (for construction of these plasmids, see Materials and Methods and Table 0.1). Proteins produced in the *in vitro* translation reactions were labelled with ^{35}S-Methionine and separated by SDS-page. Fig. 2 shows the direct visualization of RelB$_{P307}$ and RelE$_{P307}$, thus providing evidence that the corresponding genes are translated.

EXAMPLE 4

Demonstrating that *relE*$_{K-12}$ is a cytotoxin

The low copy-number cloning vector pNDM220 contains *lacIq* and the LacI regulated pA1/O4/O3 promoter (Lanzer and Bujard, 1988) upstream of a multiple cloning site (mcs). The genetic structure of pNDM220 is shown in Fig. 3. Without IPTG added to the growth medium, the pA1/O4/O3 promoter is almost completely turned off. However, with IPTG, strong transcription is induced towards the cloning site. Therefore plasmid pNDM220 is suitable for the conditional expression of genes, in particular toxin-encoding genes.

The *relE*$_{K-12}$ gene of *E. coli* K-12 (Bech et al., 1985) was PCR amplified and inserted into the mcs of pNDM220, resulting in pMG223 (for the construction of pMG223, see Materials and methods). Plasmid pMG223 (+733 to +1020) was established in MC1000, which contains a chromosomal copy of the *relBE* operon. However, it was not possible to transform pMG223 into the JS115 strain, which carries a deletion of the *relBE* operon (Δ*relB*). Therefore, the induction experiments shown in Fig. 4 were accomplished using strain MC1000, which contains the chromosomal copy of *relBE*$_{K-12}$.

Strain MC1000/pMG223 was grown in LB at 37°C. At time zero, IPTG was added to the growth-medium. After two hours of induction with IPTG, the viable counts decreased c. 600-fold (Fig. 4B). The decline started immediately and continued exponentially for about 2 hours. On plates containing IPTG, viable counts decreased even further (data not shown). The optical density (OD_{450}) increased during the first 20 minutes after addition of IPTG and then the culture became stationary (Fig. 4A). Addition of IPTG to growing cells containing the vector-plasmid had no effect (not shown). These results indicate that the *relE* gene encodes a cell toxin.

EXAMPLE 5

Demonstrating that $RelB_{K-12}$ is an antitoxin

Plasmid pMG2202 (+388 to +921) is a pBR322 derivative that contains the *relB* gene expressed from its own promoter (see Table 1.1). Plasmid pMG2201 (+388 to +597) is a pBR322 derivative that contains the *relB* promoter and the first part of $relB_{K-12}$. Thus, pMG2201 does not contain an intact *relB* gene and was included in the analyses as a control plasmid. The strains MC1000/pMG223 (pA1/O4/O3::*relE*+)-/pMG2202 ($relB^+$) and MC1000/pMG223/pMG2201 (*relB*) were subjected to a physiological growth experiment similar to the one described in Example 4. As seen from Fig. 4E and 4F, the presence of the high copy-number *relB*-carrying plasmid suppressed *relE*- dependent cell killing. The antitoxin effect was dependent on an intact *relB* reading frame, since the control-plasmid (pMG2201) carrying the promoter region and the first part of the *relB* reading frame did not prevent the *relE* mediated cell killing (Fig. 4C, 4D).

EXAMPLE 6

Demonstrating that $relE_{P307}$ encodes a very efficient cytotoxin

The medium copy number expression vector pBAD33 contains an arabinose inducible promoter (pBAD) with a multiple cloning site (mcs) and the *araC* gene (Guzman et al., 1995). The genetic structure of pBAD33 is shown in Fig. 5. Without arabinose added to the growth medium, the pBAD promoter is completely turned off. However, with

arabinose, strong transcription is induced towards the cloning site. On top of this property, the pBAD promoter is repressible by the addition of glucose to the growth medium. Thus, by the addition of glucose, transcription from pBAD can be rapidly and efficiently turned off.

The glucose repression effect is epistatic to the inducer effect by arabinose. Hence, if cells with a pBAD-carrying plasmid are grown in a medium containing both arabinose and glucose then the promoter is not induced. However, if cell-growth depletes the medium for glucose, then the promoter will be induced. Therefore, plasmid pBAD33 is suitable for the conditional turning on and off of the expression of genes, in particular toxin-encoding genes as described herein.

The *relE* gene of the *E. coli* plasmid P307 (Saul et al., 1989) was PCR amplified and inserted into the mcs of pBAD33, resulting in pHA810 (for the construction of plasmid pHA810, see Materials and methods). Thus plasmid pHA810 contains the $relE_{P307}$ gene inserted downstream of the pBAD promoter. Strain MC1000/pHA810 was grown in LB-medium without glucose at 37°C. At time zero, the culture was diluted into medium containing either 0 or 0.2% arabinose. In the arabinose-containing culture, an immediate decline in viable counts was observed (Fig. 6B, closed symbols). The decline continued exponentially throughout the experiment. After 240 min of induction with arabinose, viable counts had decreased more than five orders of magnitude. Without arabinose, cells containing pHA810 continued to grow exponentially (Fig. 6A and 6B, open symbols). On plates containing arabinose, none or very few viable cells were detected. These results show that $relE_{P307}$ gene encodes an extremely efficient cell toxin.

EXAMPLE 7

Demonstrating that $RelB_{P307}$ is an antitoxin

Plasmid pHA110 (+1 to +1122) is a pBR322 derivative that contains the $relB_{P307}$ gene expressed from its own promoter. The strain MC1000/pHA810/pHA110 ($relB_{P307}$) was subjected to a physiological growth experiment as described in Example 6. It appeared that the presence of the $relB_{P307}$-carrying plasmid pHA110 prevented

relE$_{P307}$ dependent inhibition of cell growth (Fig. 6C) and cell killing (Fig. 6D). This observation shows that *relB*$_{P307}$ codes for an antitoxin that counteracts the cell killing caused by RelE$_{P307}$.

EXAMPLE 8

Determination of the frequency of spontaneous mutants that are resistant to the killing effect of RelE

Strain MC1000/pHA810 was grown exponentially to an OD of 0.5 and serial dilutions of the cell suspension were plated on LA plates containing chloramphenicol (selecting for plasmid pHA810) and with or without 0.02% arabinose (which induces expression of *relE* present in pAH810). On such plates without arabinose the plating efficiency of strain MC1000/pAH810 was normal, i.e. more than 99% of the viable cells produced a colony. This indicated that the presence of pAH810 in itself had no effect on the viability of the cells. However, with arabinose the plating efficiency was reduced by about 109 fold, thus indicating that expression of RelE is extremely toxic to the cells. The few surviving colonies that appeared eventually were retransformed with the RelE expression plasmid pHA210 which can co-exist with pAH810. However, none of the surviving cells from the first round of selection (i.e. using pHA810) survived induction of RelE (by addition of IPTG) from the second plasmid pAH210.

These results show that resistance against RelE toxicity is a very rare event, as based on this experiment it is less than about 10^{-9}.

EXAMPLE 9

Demonstrating that RelE of the Archeon *Methanoccus janashii* is toxic to *E. coli*.

The *relE* gene of *M. jannaschii* was amplified from genomic DNA using primers MJ-relE/2CWW (5'-CCCCCGAATTCGCATGCGCCATTAGAAT, SEQ ID NO:47) and MJ-relE/1CW (5'-CCCCCGGATCCGAGCTCGAGGCTTTGAAAGAATTGGG, SEQ ID NO:48). The resulting DNA fragment was cleaved with *Bam*HI and *Eco*RI and cloned into plasmid pNDM220 (Fig. 3) thus yielding pHA705 (Fig. 7). Similarly, *relB* and *relE*

from *M. jannashii* were PCR amplified using primers relB-M.jannCW (5'-CCCCGGATC-CGTCGACGACAAATAAGGGGATAACTATG, SEQ ID NO:49) and MJ-relE/2CWW. The resulting DNA fragment was cleaved with *Bam*HI and *Eco*RI and cloned into pNDM220, thus yielding pHA715 (Fig. 7).

Plasmids pHA705 (carrying *relE*) and pHA715 (carrying *relBE*) were transformed into *E. coli* K-12 strain MC1000. Cells were grown exponentially and followed after the addition of IPTG. Fig. 8 shows that the addition af IPTG inhibited the growth of MC1000/pHA705 but not that of MC1000/pHA715, and Fig. 9 shows that viable count was significantly reduced in the case of MC1000/pHA705 but not in that of MC1000/pHA715, thus demonstrating that RelE of *M. jannashii* is toxic to *E. coli*.

EXAMPLE 10

Demonstrating that RelE of the Gram-positive bacterium *Streptococcus pneumoniae* is toxic to *E. coli*.

Using BLAST database searching, we identified two homologues of the *relBE* genes of *S. pneumoniae*. The DNA sequence of the homologue designated $relE_{Sp2}$ is shown in Fig. 10. Gene $relE_{Sp2}$ was PCR amplified from genomic DNA of *S. pneumoniae* strain RP46 using primers relE-Sp2/cw (5'-CCCCGGATCCGATGCATGATTTAGGCTTGAAG, SEQ ID NO:50) and relE-Sp2/ccw (5'-CCCCGAATTCGAATGAAA-ATTTACTTGAAAAAAG, SEQ ID NO:51). The resulting DNA fragment was cleaved with *Bam*HI and *Eco*RI and cloned into pNDM220 thus yielding plasmid pHA-Sp2 (Fig. 11).

Plasmid pHA-Sp2 (carrying $relE_{Sp2}$) was transformed into *E. coli* strain MC1000. Cells were grown exponentially and followed after the addition of IPTG. Fig. 8 shows that the addition af IPTG inhibited the growth of MC1000/pHA-Sp2, and Fig. 9 shows that viable counts were dramatically reduced, thus demonstrating that expression of $relE_{Sp2}$ is highly toxic to *E. coli*.

EXAMPLE 11

Cloning of the *relE* genes of plasmid P307, *M. jannashii* and *E. coli* K-12 into the broad-host-range vector pHAG33.

The broad-host-range vector pVLT33 is an RSF1010 derivative that can be mobilized by an appropriate conjugation system (de Lorenzo, Eltis, L., Kessler, B. and Timmis, K.N. 1993. Analysis of *Pseudomonas* gene products using *lacI*^{q}/P*trp-lac* plasmids and transposons that confer conditional phenotypes. Gene 123, 17-24). It also contains the *tac*-promoter (p*tac*) and *lacI*^{q}. Since p*tac* is leaky and therefore unsuitable for the regulated expression of toxins, the promoter was replaced by the pA1/04-03 promoter of pNDM220. The resulting plasmid, pHAG33, is shown in Fig. 12. The *relE* genes of pHA210 ($relE_{P307}$), pHA705 ($relE_{Mj}$) and pMG223 ($relE_{K12}$) were cloned into pHA33, resulting in plasmids pHA33-2 (Fig. 13), pHA33-3 (Fig. 14), and pHA33-4 (Fig. 15), respectively.

EXAMPLE 12

Demonstrating that RelEs of *E. coli* K-12, P307 and *M. jannashii* are toxic to *Pseudomonas putida*.

Plasmids pHA33-2 ($relE_{P307}$), pHA33-3 ($relE_{Mj}$) and pHA33-4 ($relE_{K12}$) were transformed into the *E. coli* K-12 strain S17-1. This strain contains the conjugation system of RP4 and is thus able to mobilize pHA33-derived plasmids as described above (Simon et al., 1986). After conjugation on solid medium to *P. putida* strain KT2440 according to standard procedure, strains KT2440/pHA33-2, KT2440/pHA33-3 and KT2440/pHA33-4 were established.

The strains were grown exponentially in LB containing 30 µg/ml ampicillin and 50 µg/ml kanamycin and followed after the addition of 2 mM IPTG. As seen from Fig. 16, the increment in cell-growth as measured by OD_{450} was reduced by IPTG in all three cases. Furthermore, measurements of viable counts (Fig. 17) showed cell-killing in all three cases, most severe in the case of $relE_{K12}$ (pHA33-4(+) in Fig. 17). Thus, RelE

proteins of P307 and *M. jannashii* are toxic to *P. putida* and RelE of *E. coli* K-12 is extremely toxic to *P. putida*.

EXAMPLE 13

Demonstrating biological containment by the depletion of a carbon source.

Plasmid pHA810 (Fig. 18) was constructed by inserting the *relE* gene of P307 into the expression vector pBAD33 (Fig. 5). The promoter (designated p_{BAD}) upstream of *relE*$_{P307}$ in pHA810 is repressed by glucose and induced by arabinose. The repression by glucose overrides induction by arabinose such that the simultaneous presence of glucose and arabinose in the growth medium results in repression of the promoter.

To simulate a realistic scenario in which the carbon source was depleted, we grew MC1000/pHA810 in ABT minimal salts medium at 35°C in the presence of a limiting amount of glucose (0.025% w/v) (represses p_{BAD}) and varying the concentration of arabinose (induces p_{BAD}). Optical density (Fig. 19) and viable counts (Fig. 20) typical for such an experiment were obtained. As seen in Fig. 19, the rate of increase in OD_{450} is severely reduced by the highest amounts of arabinose (0.050% and 0.075%). This was expected, since arabinose induces pBAD and the limited amount of glucose (0.025%) cannot fully suppress pBAD at high concentrations of arabinose. The glucose added (0.025%) was depleted by cell growth at an OD_{450} = approx. 0.1. At this OD_{450}, a dramatic cell killing was seen in the case of 0.005%, 0.010%, and 0,025% of arabinose (Fig. 20). This result shows, that depletion of the carbon source (glucose) leads to massive cell killing, and thus to biological containment of the plasmid that carries *relE*$_{P307}$.

EXAMPLE 14

Demonstrating that RelE of *E. coli* K-12 and *M. jannashii* are toxic to human cells.

The cell line 293 is a permanent line of primary human embryonal kidney cells transformed by human adenovirus type 5 (Ad 5) DNA (ATCC CRL-1573). The cells are particularly sensitive to human adenovirus, are highly permissive for adenovirus DNA, and

contain and express the transforming genes af AD 5 (Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59-74).

Plasmid pcDNA3.1(+) (Invitrogen) carries the constitutive promoter P_{CMV} from cytomegalovirus upstream of a multiple cloning site (mcs). Genes *relE* of *E. coli* K-12 and *M. jannashii* were inserted in the mcs, resulting in plasmids p5.4 and p5.3, respectively.

Plasmids pcDNA3.1(+)(control), p5.4 and p5.3 were transfected into cell line 293 by selection in medium containing G418 (geneticin), which selects for cells expressing the neomycin gene present on the plasmids. After 12 days, the cell density was measured by inspection. In the case of p5.4 ($relE_{K\text{-}12}$), between 0 and 5% of the cells had survived (as compared to the control). In the case of p5.3 ($relE_{Mj}$), between 5 and 10% of the cells had survived. These results indicate that the bacterial $RelE_{K\text{-}12}$ and the archeaeal $RelE_{Mj}$ toxins both are lethal to human cells.

EXAMPLE 15

Demonstrating that $RelE_{K\text{-}12}$, $RelE_{P307}$, and $RelE_{Mj}$ inhibit translation in vitro

DNA fragments comprising genes $relE_{K\text{-}12}$, $relE_{P307}$ and $relE_{Mj}$ were PCR amplified such that a T7 RNA polymerase promoter was placed upstream of the corresponding genes (according to Thisted et al., 1994.

The following primers were used: $relE_{K\text{-}12}$ (P1: 5'- (TGTAATACGACTCACTATA*GATAAGGAGTTTTATAA*ATGGCGTATTTTCTGGATTTTG, SEQ ID NO:52) and P2 (CACCTTCGGTGCGAAACAG, SEQ ID NO:53); $relE_{P307}$ (P3: 5'-TGTAATACGACTCACTATA*GATAAGGAGTTTTATAA*ATGAGGTATCAGGTAAAATTCA (SEQ ID NO:54) and P4: 5'-CTTTCCATCGGCGAATTATC, SEQ ID NO:55); $relE_{Mj}$ (P5: 5'-TGTAATACGACTCACTATA*GATAAGGAGTTTTATAA*ATGAAGTTTAACGTTGAGATAC SEQ ID NO:56) and P6: (5'-ATCATGGTATCAGCCGAATC, SEQ ID NO:57). T7 RNA polymerase sequences are underlined, and the strong Shine-Dalgarno (SD) sequence from the *parA* system of plasmid R1 is shown in italics.

Using in vitro transcription with T7 RNA polymerase according to standard procedures, mRNAs encoding *relE*$_{K-12}$, *relE*$_{P307}$, and *relE*$_{Mj}$ were produced and subsequently purified from a denaturing polyacrylamide gel. To facilitate the quantification of the mRNAs they were labelled with tritium (alfa-^{3}H-CTP) during their synthesis. The *relE*-encoding mRNAs (1.5 pmol) were used as templates in in vitro translation reactions employing an S30 extract (obtained from Promega) containing 150 µM of each amino acid except Methionine which was 1 µM.

Figure 21 shows SDS-PAGE (tricine-gel) analysis of such an experiment. The in vitro translation reactions were initiated with unlabelled Methionine in order to produce RelE toxin in the reaction. Ten minutes after the addition of the unlabelled Methionine, radioactive 35-S-Methionine (5 pmol in a 15 µl volume) was added and the reaction continued for an additional 20 minutes. C in Fig. 21 denotes a control lane without exogenous mRNA added. The protein bands seen in this lane originate from translation of mRNAs present in the S30 extract. In lane 1, the *in vitro* translation reaction contained an mRNA encoding the *relE* gene of *E. coli* K-12. As seen, the translation reaction was severely inhibited. In lane 2, a mRNA encoding a mutated *relE* (denoted *relEmE* and described in Gotfredsen et al, 1998) gene was added. As seen, the presence of this mRNA did not inhibit the reaction. This result shows that the RelE protein produced during the initial incubation-period without 35-S-Met added inhibits the in vitro translation reaction (i.e. compare lanes 1 & 2). Furthermore, this lack of inhibition is correlated with loss of cell killing activity in vivo (since the mutated *relE* gene, *relEmE*, used in lane 2 is not toxic to *E. coli* cells), thus indicating that inhibition of translation is the actual cause of cell death in vivo. In lanes 3 and 4, mRNAs encoding *relE* of plasmid P307 and the archaeon *M. jannashii* were added. As seen, the presence of these mRNAs inhibited the in vitro translation reactions as well. These results indicate that the RelE toxins from *E. coli* K-12, *M. jannashii* and plasmid P307 all act by inhibition of translation.

EXAMPLE 16

Demonstrating that $RelE_{K-12}$ is toxic to yeast cells.

1. Yeast strain

In these experiments the yeast strain *Saccharomyces cerevisiae* 281288DIV-36 (MATa his 4-5; LEU2 THR4 ura3-52 trp1 CYH2 KAR1) was used.

2. PCR amplification of RelE coding region

The RelE coding region was PCR amplified from the plasmid pMG223 using two ologonucleotide primers. The primer S-RelE was 24 nucleotides long (5´-TAGGTACCATGGCGTATTTTCTGG-3´, SEQ ID NO:58). It contains *Kpn*I and *Nco*I endonuclease restriction sites at the 5´end with an 8 nucleotide overhang. Primer AS-RelE was 23 nucleotides long (5'-GAGACCCCACACTACCATCGGCG-3', SEQ ID NO:59) and hybridises 400 nucleotides downstream the RelE termination codon and 392 nucleotides downstream the *Eco*RI site in plasmid vector pMG223. PCR amplifications were performed using Vent© Polymerase (New England Biolabs), 200 μM of each dNTP. PCR reaction buffer (10 mM KCl; 10 mM $(NH_4)_2SO_4$; 20 mM Tris-HCl (pH 8.8); 2 mM $MgSO_4$; 0.1% Triton X-100) with 0.2 μM of each of the primers. After 5 min denaturation (95°C) PCR was performed with 20 cycles, each cycle consisting of 1 min denaturation (92°C), 1 min primer annealing (50°C) and 1 min primer extension (72°C). Successful PCR products of 706 bp DNA fragments were identified and purified from a 1% agarose gel after a run of 1 hour at 40 mA. The PCR product was digested with the two restriction enzymes KpnI and EcoRI and the fragment of 304 bp containing the RelE open reading frame was isolated after electrophoresis on a 1.2 % agarose gel, and purified using a gel extraction kit (Pharmacia).

3. Cloning of the amplified *relE* gene

The isolated DNA fragment of the *relE* gene flanked with *Kpn*I and *Eco*RI sites was ligated into the pYES2 expression vector (Invitrogen) previously digested with *Kpn*I and *Eco*RI using standard procedures (Sambrook). After ligation, *E.coli* Top10 (Invitrogen) was transformed with the ligation mixture using electroporation using the *E.coli* gene pulser (BioRad). After phenotypic expression for 2 hours in SOC medium the culture was spread onto selective LB medium (Sambrook) containing 50 µg of ampicillin per ml. Transformed colonies were identified using PCR amplification and a positive clone designated pPK727 was further tested by restriction enzyme analysis. The functionality of the PCR amplified *RelE* gene was tested in *E coli* by cloning the *Nco*I-*Eco*RI fragment from pPK727 into the *E. coli* expression vector pUHE24. Induction with IPTG led to cell killing in *E. coli*.

4. Yeast transformation

S. cerevisiae was grown overnight (ON) in YDP medium (1% yeast extract; 2% Bacto peptone; 2 % glucose). For a single transformation, cells from 1 ml ON culture were spun down (5,000 rpm for 30 sec using an Eppendorf minicentrifuge) and washed twice in sterile water. Cells were resuspended in 200 µl lithium acetate buffer (10 mM Tris-HCl pH 7.6 with 100 mM LiOAc, 1 mM EDTA). After incubation for 15 min. at 25°C with agitation two transformations were made adding 20 µl carrier DNA (10 mg/ml salmon sperm DNA, sonicated and heat denatured) and 100 ng of the plasmids pPK727 and pYES2 (vector control), respectively. A volume of 1.2 ml 40% PEG 4,000 in 0.1 M lithium acetate buffer was added to each transformation mixture. The transformation mixtures were incubated in a 25°C incubator for 30 min before transferring to a 42°C water bath for 15 min. Cells were spun down (5,000 rpm for 30 sec.) and washed once with sterile water before plating on Uracil drop-out medium (1% Bernstein acid; 0.1% NaOH; 2% glucose; 0.67% Bacto yeast nitrogen base; 0.1% amino acids (without uracil); 2% agar).

After three days growth at 30°C single colonies were picked and streaked onto plates with Uracil drop-out medium. After two days at 30°C cells were transferred to induc-

tion medium (Uracil drop-out medium with 2% galactose as the sole carbon source) by replica-plating.

Single colonies of *S. cerevisiae* containing pPK727 and pYES2 were transferred to liquid Uracil drop-out medium (1% Bernstein acid; 0.1% NaOH; 2% glucose; 0.67% Bacto-yeast nitrogen base; 0.1% amino acids (without uracil)) and incubated ON. To compensate for difference in cell density, a volume of 50 µl per OD_{540} (optical density at 540 nm) was used to inoculate 50 ml of liquid Uracil drop-out medium with either glucose or galactose as sole carbon source, respectively. The four flasks with *S. cerevisiae* (pPK727) and *S. cerevisiae* (pYES2) in Uracil drop-out medium with or without galactose were incubated at 30°C with moderate shaking (200 rpm). To monitor growth, samples were taken at different time point and OD540 was determined. Samples were taken in duplicates and the average OD_{540} calculated and plotted against time of sampling

5. Results.

All colonies containing either the plasmid pPK727 or the pYES2 control plasmid were able to grow on plates with glucose as carbon source. When transferred to plates with galactose as the sole carbon source leading to gene expression from the P-gal1 promoter only cells with the pYES2 control plasmid showed normal growth, whereas cells containing the pPK727 were strongly inhibited in growth.

In liquid media, the yeast cells in which the *relE* gene was induced showed a remarked growth inhibition when compared to the uninduced control and to the controls with only the plasmid pYES2 without insert.

These results that are summarised in the below Table 16.1 clearly suggest that inhibition of cell growth in yeast cells is due to expression of the *relE* gene.

Table 16.1. Growth of *Saccharomyces cerevisiae* transformed with pYES2 +/- *relE* gene

time (hours)	Plasmid + relE		plasmid (control)	
	Galactose	no galactose	galactose	no galactose
0	0	0	0	0
5,5	0,018	0,024	0,023	0,028
15,5	0,013	0,186	0,055	0,176
23	0,190	1,195	0,286	0,963
28	0,010	1,480	0,816	1,649
29	0,035	3,930	1,660	2,650
65	0,990	3,950	6,180	3,820

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CLAIMS

1. A method of conditionally controlling the survivability of a recombinant microbial cell population, the method comprising (i) providing in the cells of said population a gene coding for a cytotoxic first kind of polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally equivalent polypeptide (said genes collectively being designated herein as the *relE* gene family), said gene is expressible in the cells of the population and, operably linked to the gene, a regulatable regulatory DNA sequence and (ii) cultivating the cell population under conditions where the *relE* gene or the gene coding for a functionally equivalent polypeptide is expressed, the expression leading to an at least partial killing of the cell population.

2. A method according to claim 1 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium.

3. A method according to claim 2 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp., *Pseudomonadaceae* spp., *Helicobacter* spp. and *Synechosystis* spp.

4. A method according to claim 1 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium.

5. A method according to claim 4 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.

6. A method according to claim 5 wherein the Gram-positive bacterium is *Bacillus thuringiensis*.

7. A method according to claim 1 wherein the gene coding for the functionally equivalent polypeptide is derived from a species belonging to *Arhae*.

8. A method according to claim 1 wherein the *relE* family gene is present on the chromosome of the cells.

9. A method according to claim 8 wherein the cells of the population do not contain a gene coding for a second type of polypeptide that is capable of counteracting the cell toxic effect of the RelE polypeptide or the functional equivalent hereof.

10. A method according to claim 1 wherein the gene coding for the RelE polypeptide or the functional equivalent hereof is present on an extrachromosomal replicon.

11. A method according to claim 1 wherein the regulatory DNA sequence regulates the expression of the gene coding for the RelE polypeptide or the functionally equivalent polypeptide at the transcriptional level by means of a promoter, the function of which is regulated by the presence or absence of a chemical compound in the cultivation medium.

12. A method according to claim 11 wherein the promoter is inducible by a chemical compound.

13. A method according to claim 11 wherein the promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound whereby, when the first kind of compound is depleted from the medium, the promoter is induced by the second kind of compound.

14. A method according to claim 1 wherein the cells of the population comprises a gene coding for a second kind of polypeptide that is capable of binding to the *relE* polypeptide or the functional equivalent, said binding resulting in that the toxic effect of the RelE polypeptide or the functional equivalent is at least partially counteracted.

15. A method according to claim 14 wherein the gene coding for the second kind of polypeptide is operably linked to a regulatable regulatory DNA sequence, permitting that the gene coding for the second kind of polypeptide is suppressed under conditions where the gene coding for the RelE polypeptide or the functional equivalent is expressed.

16. A method according to claim 14 wherein the second kind of polypeptide is the RelB polypeptide derived from *E. coli* K-12 or a functionally equivalent polypeptide.

17. A method according to claim 16 wherein the functionally equivalent second kind of polypeptide is derived from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae* spp.

18. A method according to claim 1 wherein the expression of the *relE* gene family gene is stochastically regulated.

19. A method according to claim 18 wherein the stochastical regulation is effected by operably linking the *relE* gene family gene to a regulatory sequence that comprises an invertible promoter.

20. A method according to claim 18 wherein the stochastical regulation is effected by flanking at least part of the regulatory sequence by repeat sequences whereby the at least part of the regulatory sequence is recombinationally excised.

21. A method according to claim 1 wherein the cells contain a gene coding for a gene product of interest.

22. A method according to claim 21 wherein the gene product of interest is an immunologically active gene product.

23. A method according to claim 21 wherein the gene product of interest is one that is effective in degradation of an environmental pollutant.

24. A method according to claim 21 wherein the gene product of interest is a pesticidally active product.

25. A method according to claim 24 wherein the gene coding for the pesticidally active gene product is derived from *Bacillus thuringiensis*.

26. A method of confining an extrachromosomal replicon to a microbial cell population, the method comprising the steps of

(i) isolating a microbial cell naturally containing a gene belonging to the *relE* gene family coding for a first kind of polypeptide that, when it is expressed in the cell, acts as a toxin for the cell or, if the cell does not naturally contain a gene belonging to the *relE* gene family, introducing such a gene into the cell,

(ii) introducing into the cell the extrachromosomal replicon to be confined, said replicon containing a gene coding for a second kind of polypeptide that, by binding to the first kind of polypeptide, acts as an antitoxin for said first kind of polypeptide,

(iii) cultivating the cell under conditions where the genes coding for the first and the second kind of polypeptides are expressed, whereby a daughter cell that does not receive a copy of the extrachromosomal replicon is killed by the first kind of polypeptide being expressed in the absence of expression of the second kind of polypeptide.

27. A method according to claim 26 wherein the cell population consists of cells that comprises a gene coding for a gene product of interest.

28. A method according to claim 27 wherein the gene product of interest is selected from the group consisting of an enzyme, an immunologically active polypeptide, a pesticidally active gene product and a pharmaceutically active gene product.

29. A method according to claim 26 wherein the replicon is a plasmid occurring in the microbial cells at a copy number which is in the range of 1-30 including the range of 1-10 such as the range of 1-5.

30. A method according to claim 26 wherein the microbial cells belong to a Gram-negative bacterial species.

31. A method according to claim 30 wherein the bacterial species is selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp.

32. A method according to claim 26 wherein the microbial cells belong to a Gram-positive bacterial species.

33. A method of post-segregationally stabilizing a plasmid in a microbial host cell population, the method comprising the steps of

(i) recombinationally inserting into the plasmid (a) a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a functional equivalent thereof, said first kind of polypeptide having a toxin effect on the host cell and (b) a gene coding for a second kind of polypeptide that (1) is capable of acting as an antitoxin for first kind of polypeptide and (2) is capable of being degraded in the host cell at a higher rate than that at which the first kind of polypeptide is degraded,

(ii) cultivating the cell population under conditions where the genes coding for the first kind and second kind of polypeptides are expressed,

whereby a daughter cell that does not receive at least one copy of the plasmid is killed as a result of the faster degradation of the second kind of polypeptide.

34. A method according to claim 33 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium.

35. A method according to claim 34 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp., *Pseudomonadaceae* spp., *Helicobacter* spp. and *Synechosystis* spp.

36. A method according to claim 33 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium.

37. A method according to claim 36 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.

38. A method according to claim 37 wherein the Gram-positive bacterium is *Bacillus thuringiensis*.

39. A method according to claim 33 wherein the gene coding for the functionally equivalent polypeptide is derived from a species belonging to *Arhae*.

40. A method according to claim 33 wherein the second kind of polypeptide is selected from the group consisting of the relB polypeptide derived from *E. coli* K-12 and a functionally equivalent polypeptide.

41. A method according to claim 40 wherein the functionally equivalent second kind of polypeptide is derived from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae* spp.

42. A method according to claim 33 wherein the plasmid contains a gene coding for a gene product of interest.

43. A method according to claim 42 wherein the gene product of interest is an immunologically active gene product.

44. A method according to claim 42 wherein the gene product of interest is one that is effective in degradation of an environmental pollutant.

45. A method according to claim 42 wherein the gene product of interest is a pesticidally active product.

46. A method according to claim 45 wherein the gene coding for the pesticidally active gene product is derived from *Bacillus thuringiensis*.

47. A method according to claim 33 wherein the microbial cells belong to a Gram-negative bacterial species.

48. A method according to claim 47 wherein the bacterial species is selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp.

49. A method according to claim 33 wherein the microbial cells belong to a Gram-positive bacterial species.

50. A method according to claim 33 wherein the plasmid is one that occurs in the microbial cells at a copy number which is in the range of 1-30 including the range of 1-10 such as the range of 1-5.

51. A recombinant cell comprising a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally equivalent polypeptide or a variant or derivative hereof, said first kind of polypeptide having a toxic effect on the cell, subject to the limitation that when the cell is *E. coli*, the gene coding for the first kind of polypeptide is not derived from *E. coli*.

52. A cell according to claim 51 wherein the cells belong to a bacterial species selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp.

53. A cell according to claim 51 wherein the cells belong to a Gram-positive bacterial species.

54. A cell according to claim 51 wherein the gene coding for *E. coli* K-12 RelE polypeptide or the functional equivalent hereof is located on the chromosome.

55. A cell according to claim 51 wherein the gene coding for the *E. coli* K-12 RelE polypeptide or the functional equivalent hereof is located on an extrachromosomal replicon.

56. A cell according to claim 51 which comprises at least one gene coding for a gene product of interest.

57. A cell according to claim 56 wherein the gene product of interest is an immunologically active gene product.

58. A cell according to claim 56 wherein the gene product of interest is one that is effective in degradation of an environmental pollutant.

59. A cell according to claim 51 wherein the gene product of interest is a pesticidally active product.

60. A cell according to claim 51 which comprises a gene coding for a second kind of polypeptide that is capable of binding to the first kind of polypeptide whereby the toxic effect hereof is at least reduced.

61. A cell according to claim 51 comprising, operably linked to the gene coding for a first kind of polypeptide, a regulatable regulatory DNA sequence.

62. A cell according to claim 61 wherein the regulatory DNA sequence regulates the expression of the gene coding for the first kind of polypeptide at the transcriptional level by means of a promoter, the function of which is regulated by the presence or absence of a chemical compound in the cultivation medium.

63. A cell according to claim 62 wherein the promoter is inducible by a chemical compound.

64. A cell according to claim 62 wherein the promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound whereby, when the first kind of compound is depleted from the medium, the promoter is induced by the second kind of compound.

65. A cell according to claim 51 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium.

66. A cell according to claim 65 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium selected from the group

consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp., *Pseudomonadaceae* spp., *Heliobacter* spp. and *Synechosystis* spp.

67. A cell according to claim 51 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium.

68. A cell according to claim 67 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.

69. A cell according to claim 68 wherein the Gram-positive bacterium is *Bacillus thuringiensis.*

70. A cell according to claim 51 wherein the gene coding for the functionally equivalent polypeptide is derived from a species belonging to *Arhae*.

71. A cell according to claim 51 which does not contain a gene coding for a second type of polypeptide that is capable of counteracting the cell toxic effect of the first kind of polypeptide.

72. A cell according to claim 51 which comprises a gene coding for a second kind of polypeptide that is capable of binding to the first kind of polypeptide, said binding resulting in that the toxic effect of the first kind of polypeptide is at least partially counteracted.

73. A cell according to claim 72 wherein the gene coding for the second kind of polypeptide is operably linked to a regulatable regulatory DNA sequence, permitting that the gene coding for said second kind of polypeptide is suppressed under conditions where the gene coding for the RelE polypeptide or the functional equivalent is expressed.

74. A cell according to claim 72 wherein the second kind of polypeptide is the RelB polypeptide derived from *E. coli* K-12 or a functionally equivalent polypeptide.

75. A cell according to claim 74 wherein the functionally equivalent second kind of polypeptide is derived from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae* spp.

76. A cell according to claim 61 wherein the expression of the gene coding for the first kind of polypeptide is stochastically regulated.

77. A cell according to claim 76 wherein the stochastical regulation is effected by operably linking the gene coding for the first kind of polypeptide to a regulatory sequence that comprises an invertible promoter.

78. A cell according to claim 76 wherein the stochastical regulation is effected by flanking at least part of the regulatory sequence by repeat sequences whereby the at least part of the regulatory sequence is recombinationally excised.

79. A composition comprising a cell according to any of claims 51-78.

80. A method of limiting the survival of a cell population in a first or a second environment, which method comprises

(i) transforming the cells of said population with a gene coding for a cytotoxic polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide, the gene coding for the prophage P1 Doc polypeptide and a gene coding for a functionally equivalent polypeptide for anyone of said polypeptides, said gene is expressible in the cells of the population, and operably linked to the gene, a regulatory DNA sequence being regulatable by an environmental factor and which regulates the expression of said gene, and

(ii) cultivating the cell population under environmental conditions where the gene coding for the cytotoxic polypeptide is expressed, the expression leading to an at least partial killing of the cell population.

81. A method according to claim 80 wherein the survival of the cell population is limited in a first environment in which the gene is expressed, said cell population thereby being contained in said first environment.

82. A method according to claim 80 wherein the survival of the cell population is not limited when present in a first environment, which first environment could change to a second environment physically and/or chemically distinct from the first environment, in which first environment the gene whose expression results in the formation of a cytotoxically active polypeptide is not expressed, but the survival of which cell population is limited when transferred to a second environment or when present in a physically and/or chemically changed first environment, where the gene is expressed.

83. A method according to claim 80 wherein the survival of a cell population is being limited by providing in the cells a gene coding for a cytotoxic polypeptide which is operably linked to a nucleotide sequence encoding an antitoxin repressor substance which can undergo a decay when said cells are released to the outer environment to an extent whereby the repressor substance is converted to a non-functional form, whereby as a result of said decay, the function of the cells of the population will be gradually limited.

84. A method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is naturally transferable to a second kind of cell, which method comprises providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides to an extent whereby the function of the cell is being limited, said first kind of cells having or being modified to have a chromosomal replicon comprising a regulatory nucleotide sequence the gene product of which inhibits the expression of said gene or the cell function-limiting effect of the polypeptide and thereby protects said first kind of cells, said regulatory gene being lacking in said second kind of cell, whereby, if a cell of the second kind receives said extrachromosomal recombinant replicon said gene is expressed and has a function-limiting effect on said second kind of cell.

85. A method according to claim 84 wherein the gene product which inhibits the expression of the expression of the gene coding for the polypeptide or the cell function-limiting effect of the polypeptide is selected from the *E. coli* relB polypeptide, the plasmid F CcdA polypeptide, the plasmid R1 Peml polypeptide, the plasmid RP4 ParD polypeptide, the prophage P1 Phd polypeptide and a functionally equivalent polypeptide of anyone of such polypeptides.

86. A method of stochastically limiting in an environment the survival of a cell population, the method comprising transforming the cells thereof with a recombinant replicon containing a regulatably expressible gene which, when expressed in a cell encodes a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides, the expression of said gene leading to formation of the polypeptide to an extent whereby the function of the cells is being limited, the expression of said gene is stochastically induced as a result of recombinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cells, inhibits expression of the gene coding for the polypeptide, said negatively functioning regulatory nucleotide sequence being contained in the recombinant replicon or in an other recombinant replicon present in cells of the population containing the replicon.

87. A cell according to claim 51 that is an animal cell.

88. A cell according to claim 87 that is selected from the group consisting of a mammal cell, a human cell and an insect cell.

89. A method according to any one of claims 1, 26, 32, 80, 84 or 86 wherein the first kind of polypeptide inhibits translation.

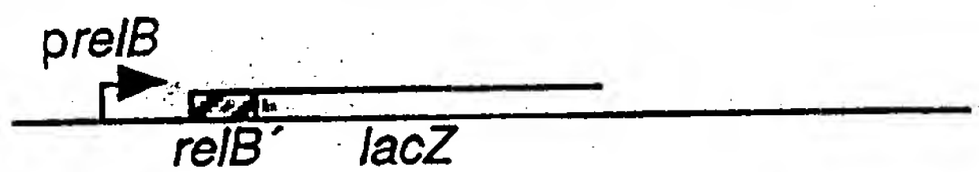
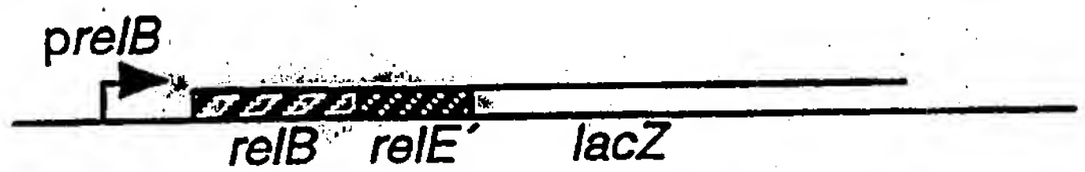
Plasmids	*relBE* derived fragments	β-galactosidase activity	Fold of repression
pKG4001		1250	1
pKG4002		28	45

Fig. 1

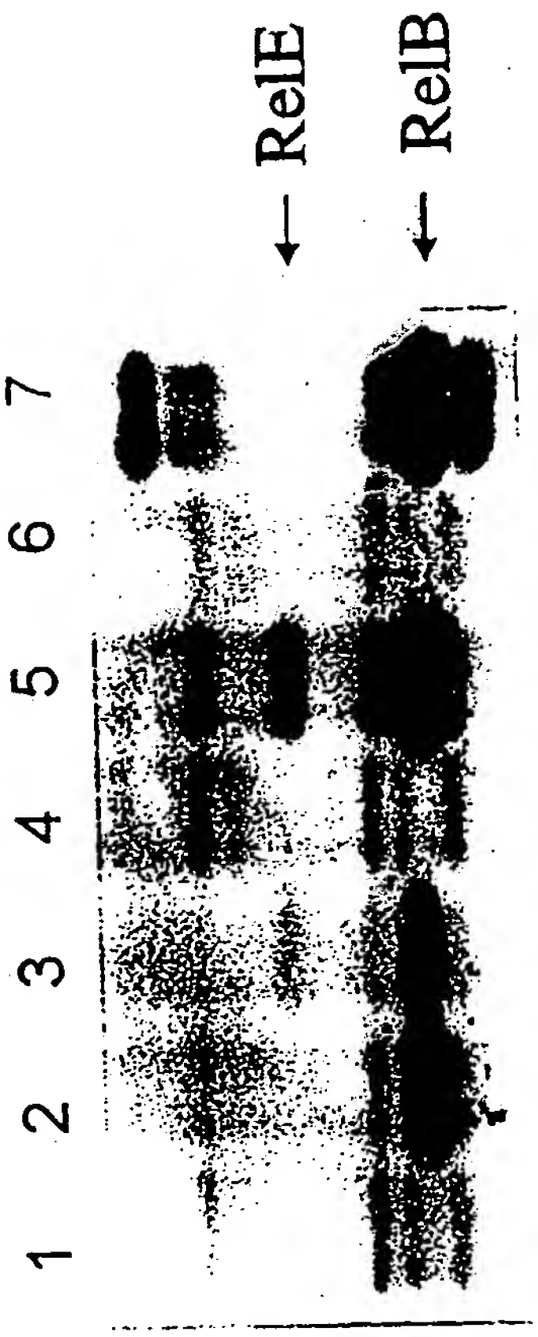


Fig. 2

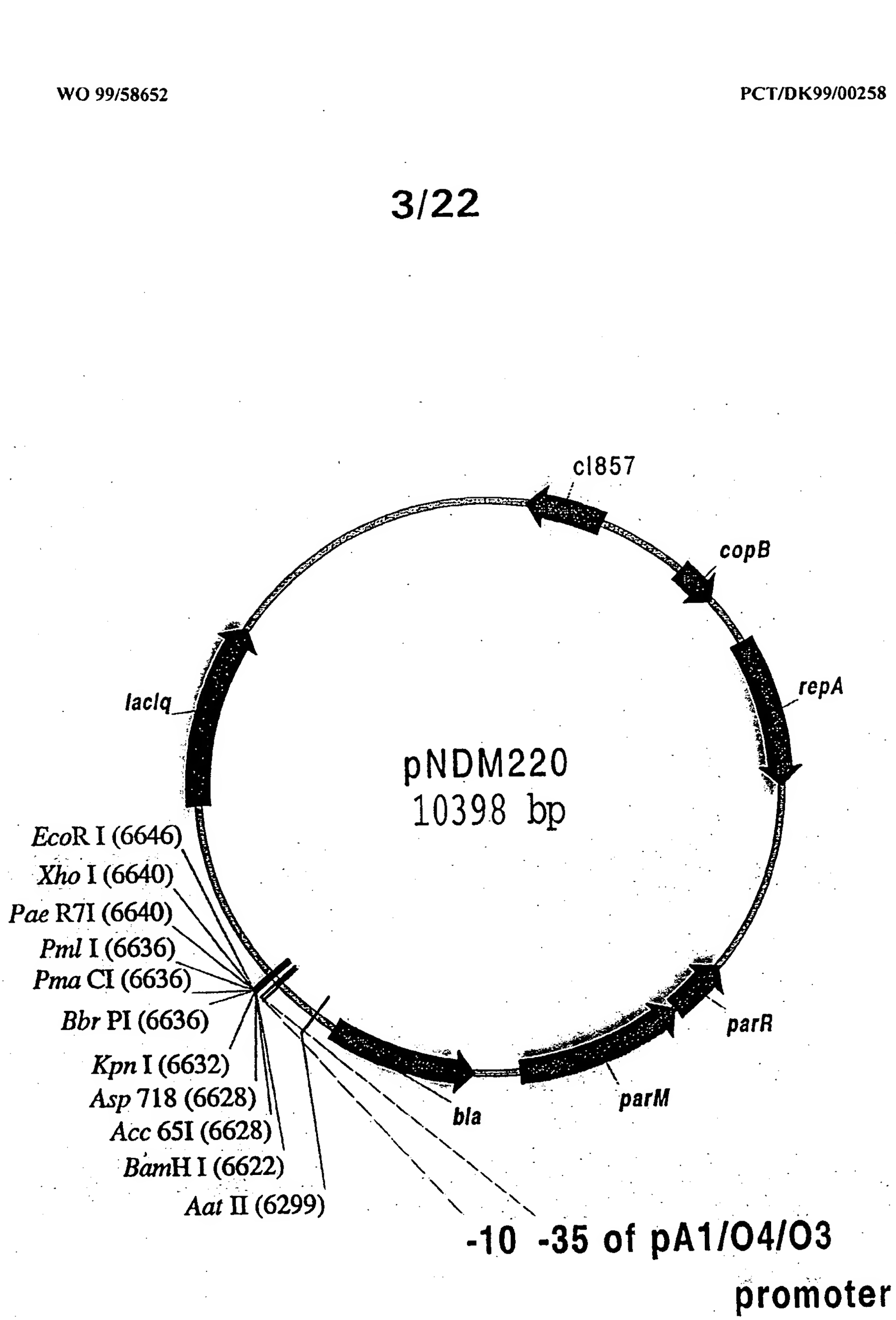

cI857
copB
repA
lacIq
pNDM220
10398 bp
EcoR I (6646)
Xho I (6640)
Pae R7I (6640)
Pml I (6636)
Pma CI (6636)
Bbr PI (6636)
Kpn I (6632)
Asp 718 (6628)
Acc 65I (6628)
BamH I (6622)
Aat II (6299)
bla
parM
parR
-10 -35 of pA1/O4/O3
promoter

Fig. 3

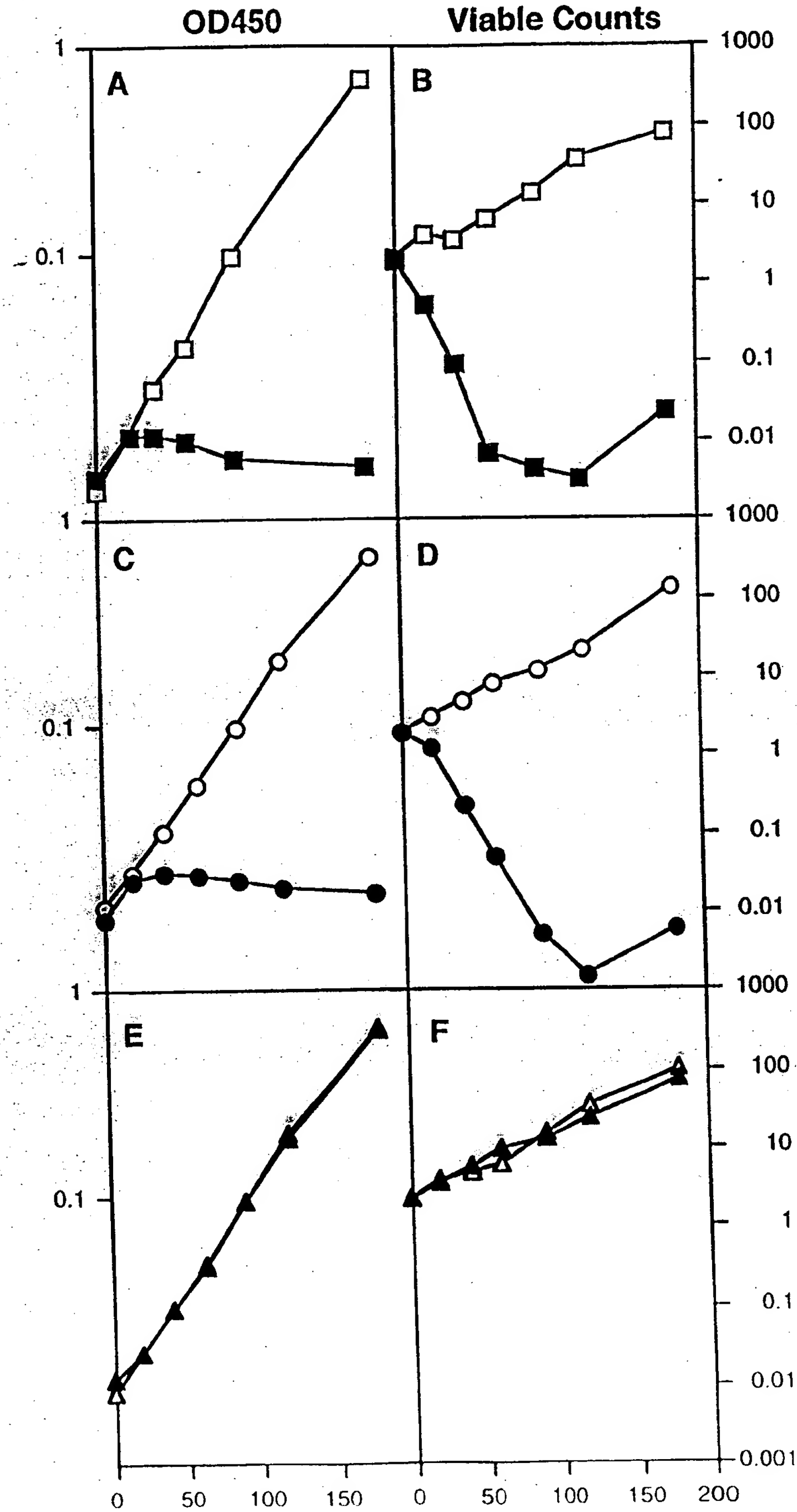

OD450
Viable Counts
A
B
C
D
E
F
1
0.1
1
1000
100
10
1
0.1
0.01
1000
100
10
1
0.1
0.01
1000
100
10
1
0.1
0.01
0.001
0
50
100
150
0
50
100
150
200

Fig. 4

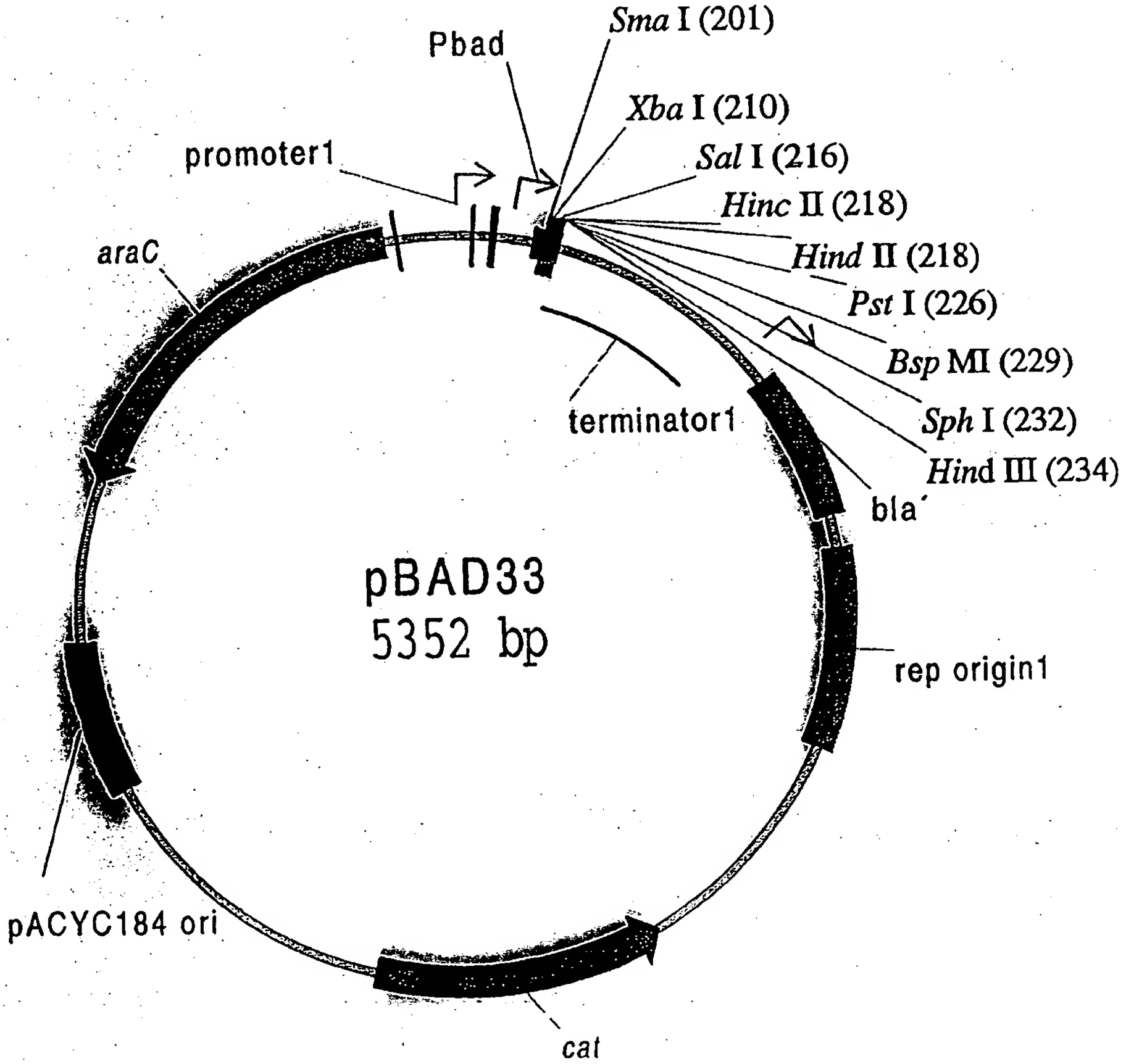
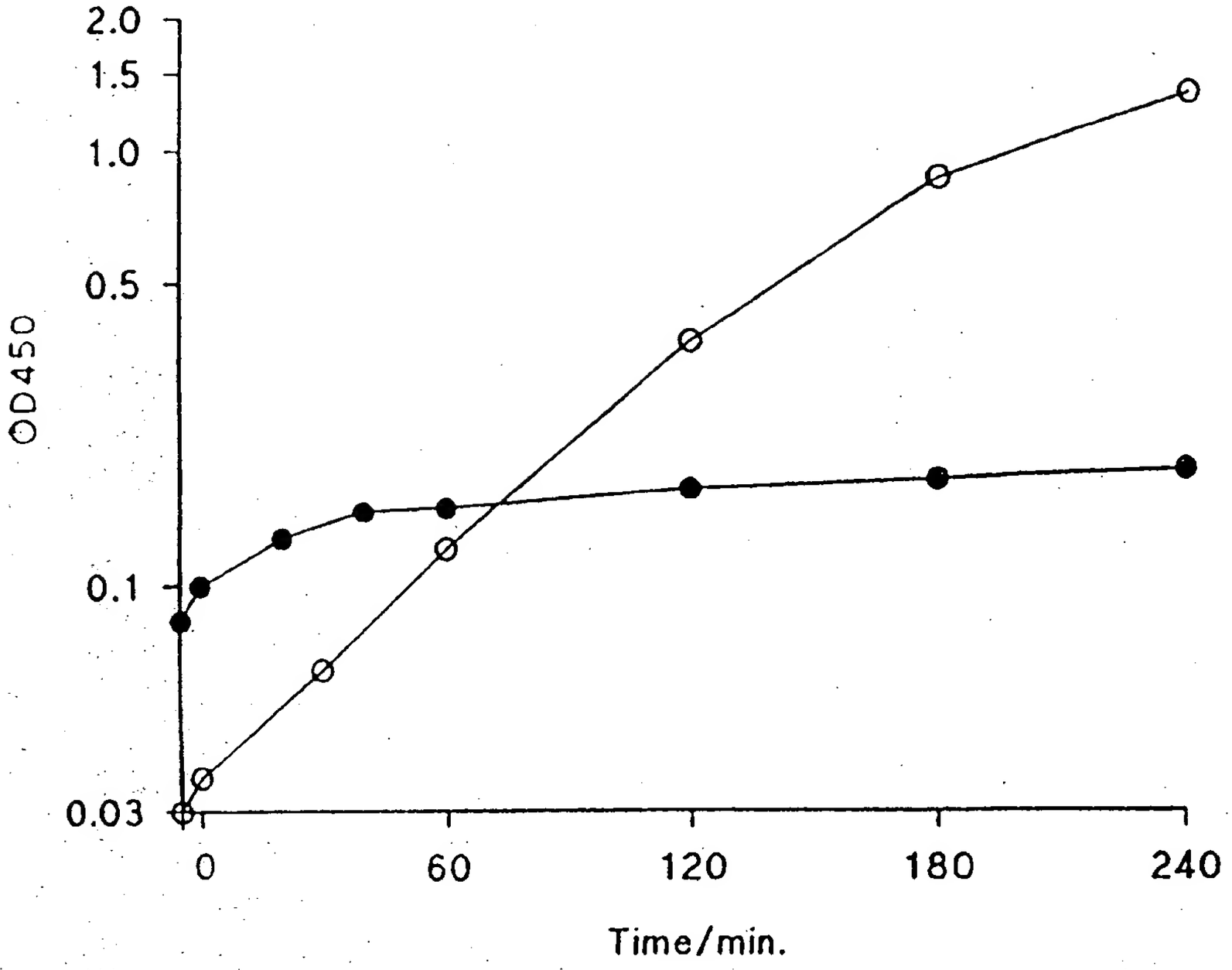

Pbad
Sma I (201)
Xba I (210)
Sal I (216)
Hinc II (218)
Hind II (218)
Pst I (226)
Bsp MI (229)
Sph I (232)
Hind III (234)
promoter1
araC
terminator1
bla
pBAD33
5352 bp
rep origin1
pACYC184 ori
cat

Fig. 5

A


2.0
1.5
1.0
0.5
0.1
0.03
OD450
0
60
120
180
240
Time/min.

B

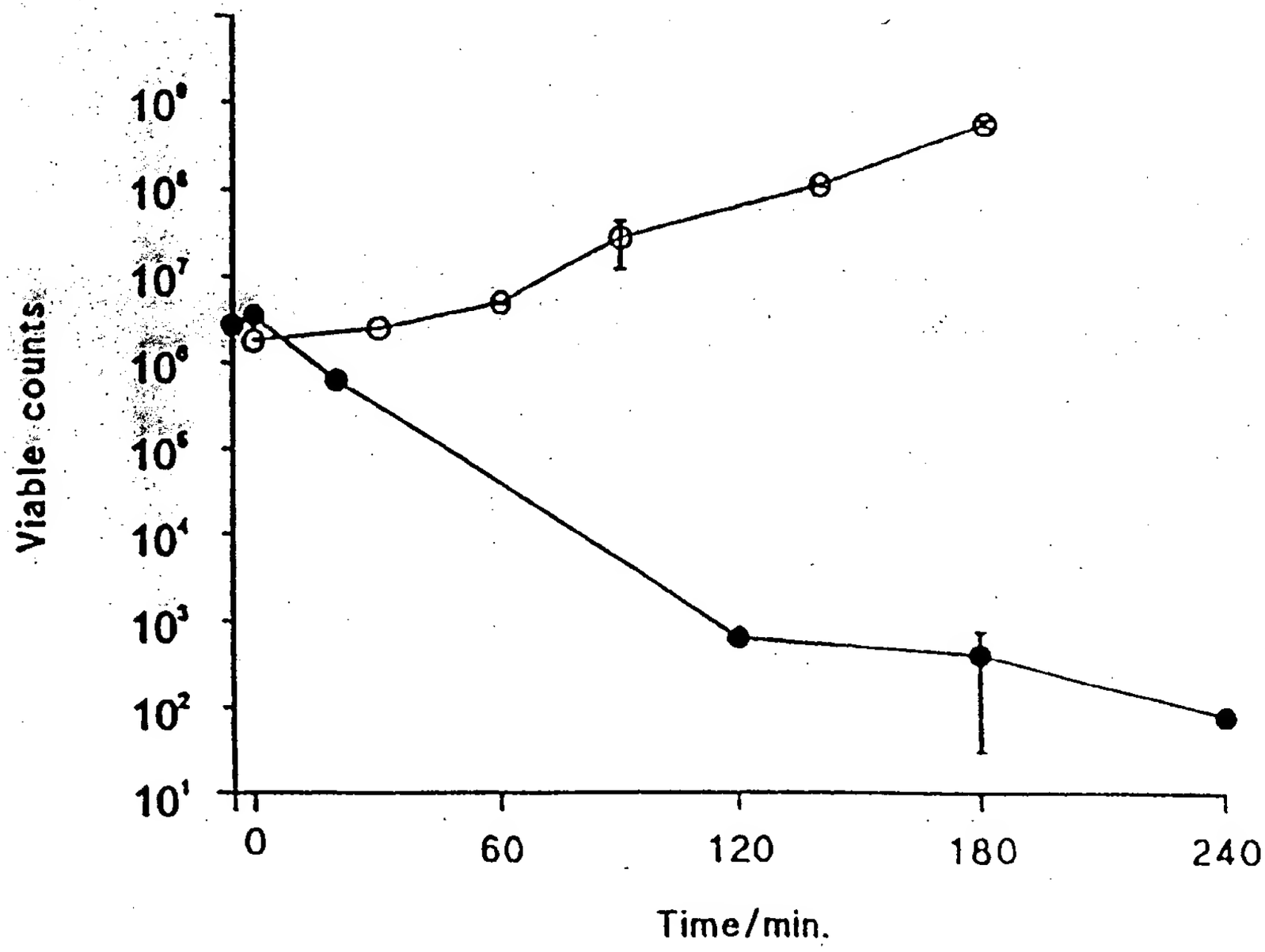

Viable counts
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
60
120
180
240
Time/min.

Fig. 6A

C 7/22

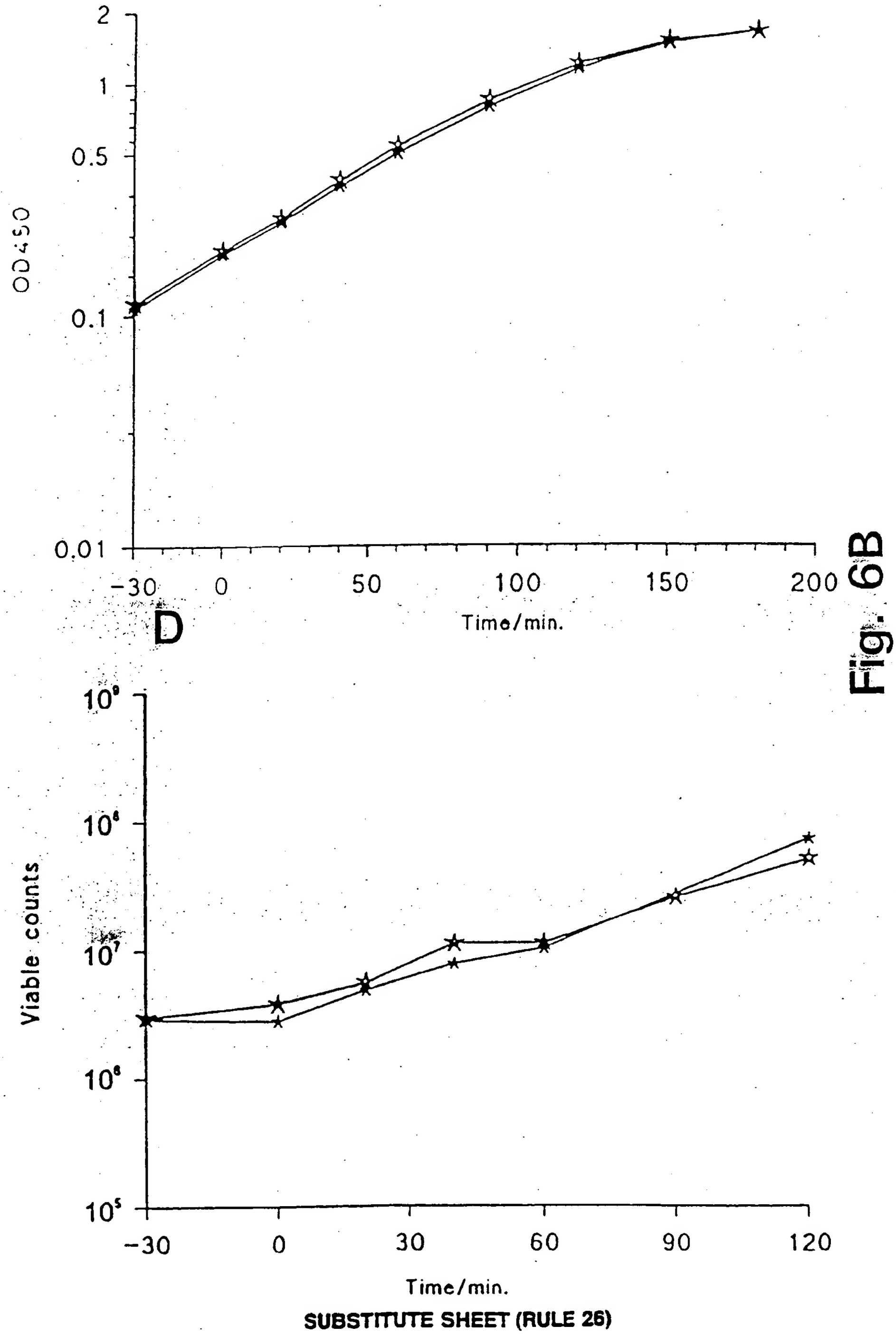

2
1
0.5
0.1
0.01
OD450
-30
0
50
100
150
200
Time/min.
D
10⁹
10⁸
10⁷
10⁶
10⁵
Viable counts
-30
0
30
60
90
120
Time/min.

Fig. 6B

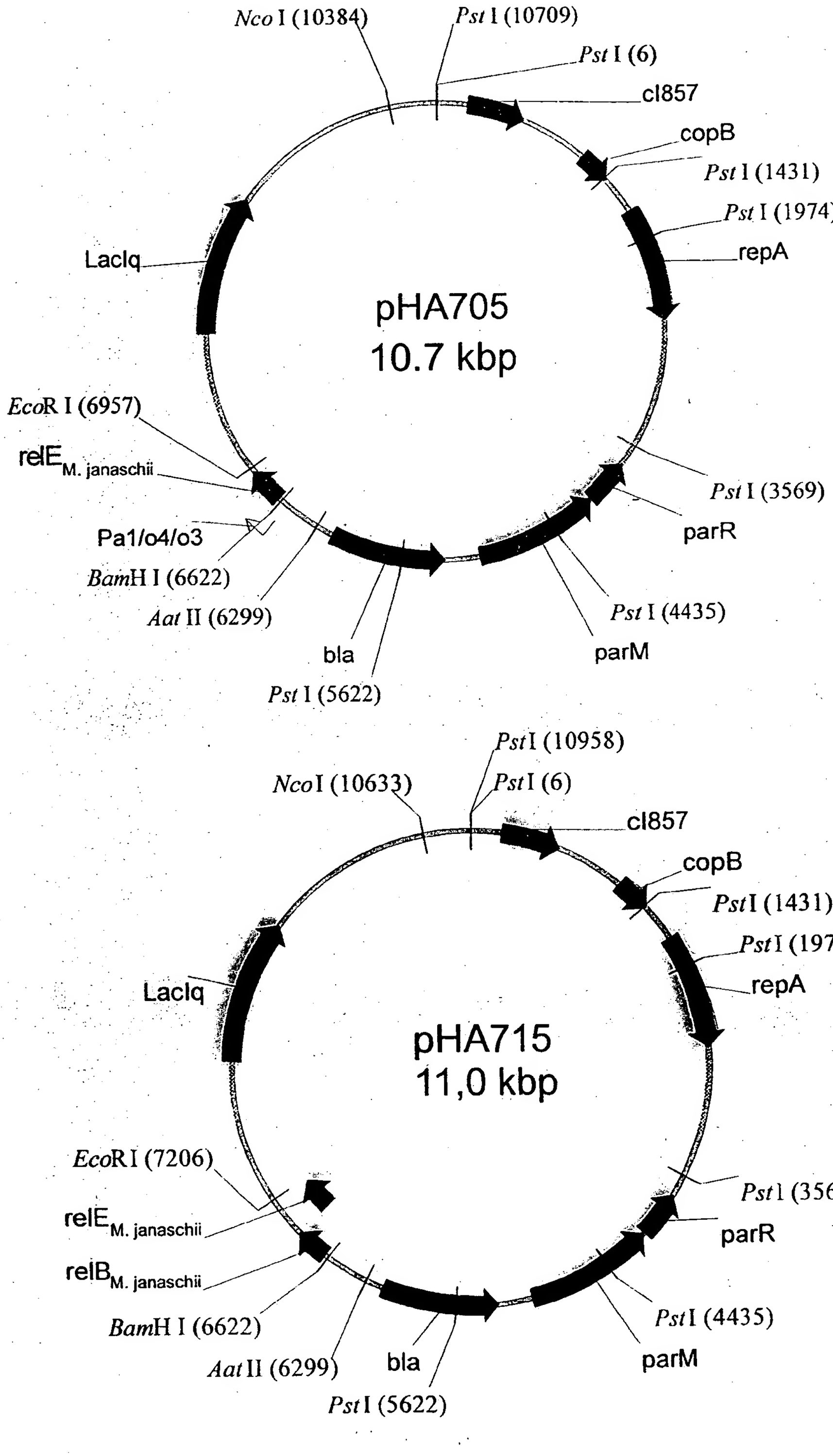

NcoI (10384)
PstI (10709)
PstI (6)
cI857
copB
PstI (1431)
PstI (1974)
repA
LacIq
pHA705
10.7 kbp
EcoRI (6957)
relE M. janaschii
Pa1/o4/o3
BamHI (6622)
AatII (6299)
bla
PstI (5622)
PstI (3569)
parR
PstI (4435)
parM
PstI (10958)
NcoI (10633)
PstI (6)
cI857
copB
PstI (1431)
PstI (1974)
repA
LacIq
pHA715
11,0 kbp
EcoRI (7206)
relE M. janaschii
relB M. janaschii
BamHI (6622)
AatII (6299)
bla
PstI (5622)
PstI (3569)
parR
PstI (4435)
parM

Fig. 7

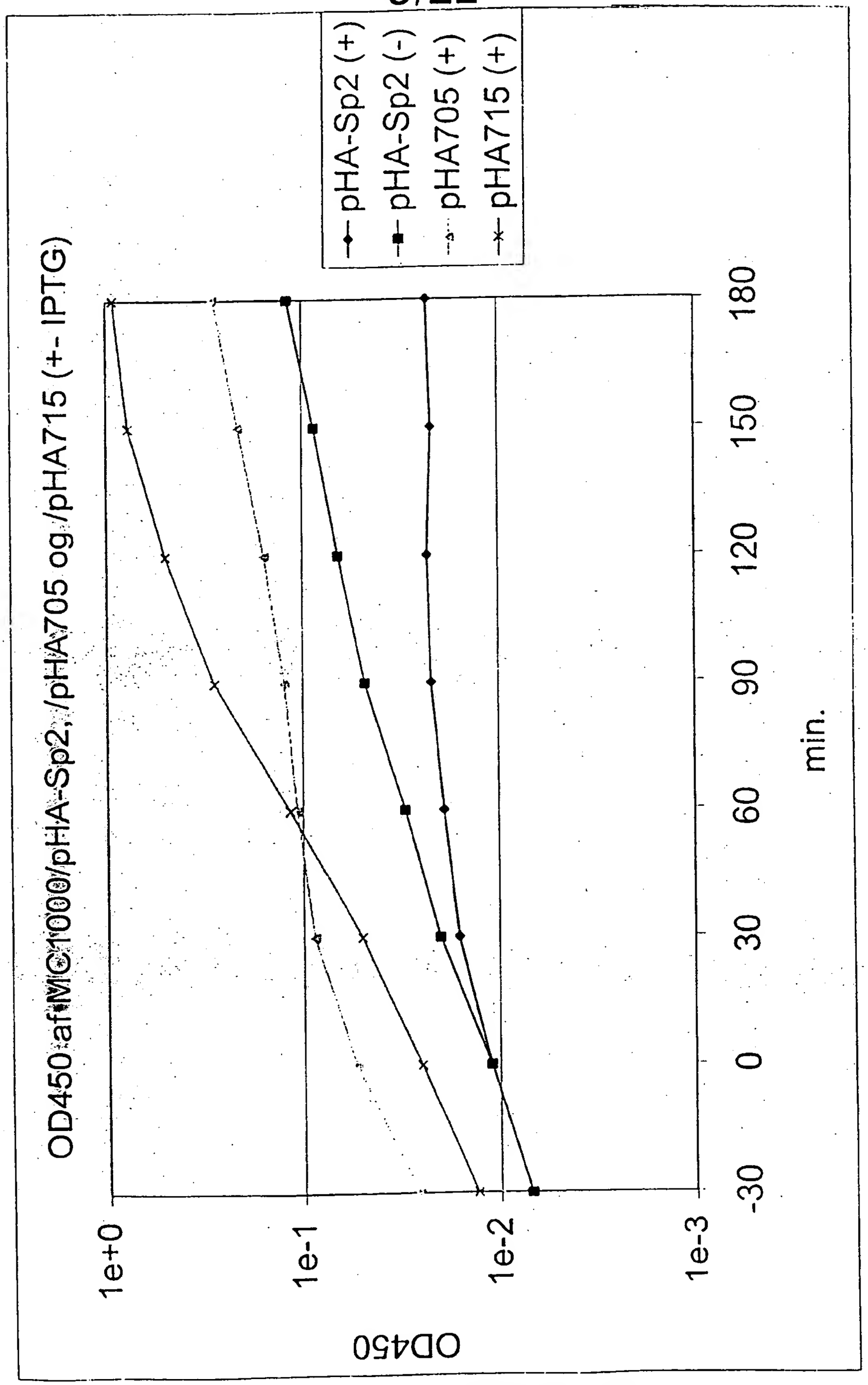

pHA-Sp2 (+)
pHA-Sp2 (-)
pHA705 (+)
pHA715 (+)
OD450 af MIC1000/pHA-Sp2, /pHA705 og /pHA715 (+- IPTG)
OD450
1e+0
1e-1
1e-2
1e-3
-30
0
30
60
90
120
150
180
min.

Fig. 8

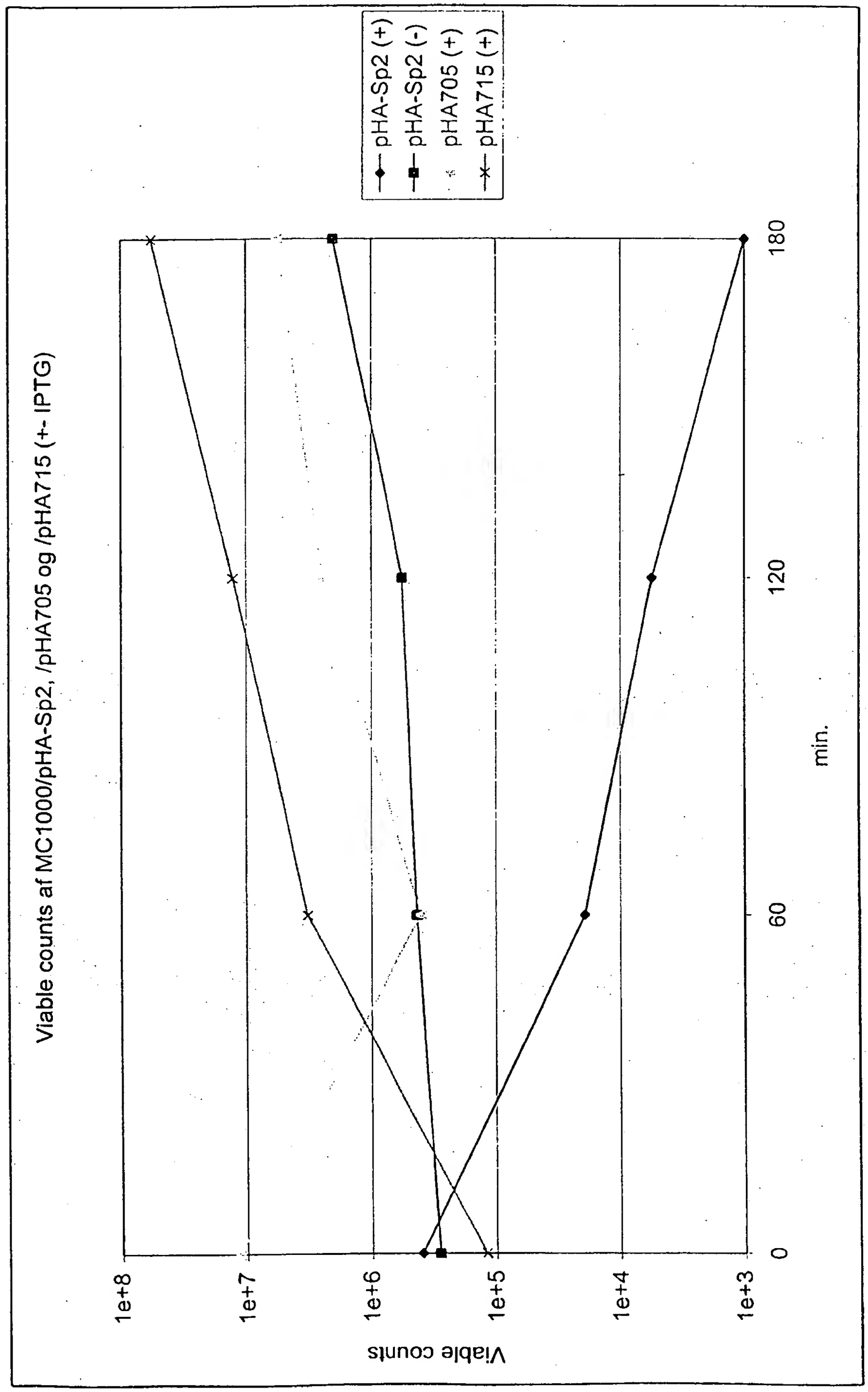

pHA-Sp2 (+)
pHA-Sp2 (-)
pHA705 (+)
pHA715 (+)
Viable counts af MC1000/pHA-Sp2, /pHA705 og /pHA715 (+- IPTG)
Viable counts
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
0
60
120
180
min.

Fig. 9

DNA sequence of the *relBE*$_{Sp2}$ locus of *S. pneumoniae* (reverse complement of part of contig 80 in the tigr database)

```
   1  ACCCATGAGC ATTCAGATCA TATCCATGGA GTAGGCGTTT TGGCTCGCAA
  51  GTATGGTATG GATCTTTATG CCAATGAAAA GACCTGGCAA GCTATGGAAA
 101  ATAGTAAATA TCTTGGCAAG GTGGATTCTT CGCAAAAGCA TATTTTTGAA
 151  ATGGGTAAAA CCAAAACCTT TGGAGATATC GACATCGAGA GTTTTGGTGT
 201  AAGCCATGAT GCAGTCGCAC CGCAGTTCTA TCGCTTTATG AAGGATGATA
 251  AGAGTTTTGT CCTCTTGACA GATACAGGTT ATGTCAGTGA CCGTATGGCG
 301  GGCATTGTCG AAAATGCGGA TGGTTATCTT ATCGAGGCCA ACCATGATGT
 351  AGAGATTTTG CGATCAGGTT CTTACGCTTG GCGACTCAAA CAACGAATCC
 401  TATCTGACCT TGGTCACCTT TCTAACGAGG ACGGTGCTGA AGCTATGATT
 451  CGGACGCTAG GAAATCGTAC TAAGAAGATT TACCTTGGGC ATTTATCTAA
 501  GGAAAACAAT ATCAAGGAAC TGGCTCATAT GACCATGGTC AATCAGCTGG
 551  CTCAAGCTGA TCTGGGAGTC GGAGTAGACT TTAAGGTTTA TGATACCTCA
 601  CCAGATACCG CAACACCATT GACAGAGATA TAGAAAGAAC GCTGAGAAGG
 651  TGTTCTTTTT ATATTGACTG AACACCTAAA AAGTAATACA ATGGTGTTAC
                SD                Start relB_Sp2
 701  CATTAAAAAA GGGAGCACAA AAGATGACTA CTATTACATT AAAAGTTTCT
 751  GAAGCTGATA AAACATTTAT GAAAGCAATG GCTAAGTTTG AAGGAGTTTC
 801  CCTTTCGGAA CTTATTCGCA CCAAAACTCT TGAAGCCCTA GAAGATGAAT
 851  ACGATGCTCG TGTGGCAGAT TTAGCCTATC AAGAGTATTT AGAAGACTTG
                                                       SD
 901  GAAAAAGGAG TTGAACCCAT TACTTGGGAA GAAATGATGC ATGATTTAGG
      Start relE_Sp2/End relB_Sp2
 951  CTTGAAGGAT GAATAATTTG TATAAATTAG TTCCAACAAG ACGTTTTATC
1001  AAGCAATTGA AAAAATTGGA CCGTTATACG CAGAAGCTAA TTACAAACTA
1051  TTTACAAACC AATGTTTTGG AAGACCCAAG ACGACACGGA AAGGCTTTGG
1101  TTGGTAATCG CGTTGGTCAA TGGCGCTATA GAATTGGTAA TTATCGAGTT
1151  ATCGTACAAA TTGTAGATGA TGAATTAGTC GTTGCTACTC TAGAAGTTGG
                        End relE_Sp2
1201  TCATCGGAGA GATATTTATT GAATTACTTT TTTCAAGTAA ATTTTCATTC
1251  TTTAAAATAT GATATAATAG GCTTACAAT ATAAGAGAGG TTGCTGATGA
1301  CAATAGATAT TAGTGAAGAA AGTTTGGCTA AAGAATCGGC TGACTTATTA
1351  AAAATTCTTT TAAAAGATCG AACGACAAAG AAATCAATTG TTTGGGCTAC
1401  GCATTCTTAT GAATTGTTAG GAAAGGGATT TGCTCCAAGT GATCGCATCA
1451  ATCCAAGTAA GGTTACTGGA AACTTTGCCA ACTTGATTCA GCCCAGATCT
1501  GAAAAATCCA AATATGAACA AAAAGATCGG ACAAAAATAA GGGCAGAAGT
```

Protein sequences:

RelB$_{Sp2}$ (pHi=4.2):
mttitlkvse adktfmkama kfegvslsel irtktleale deydarvadl ayqeyledle
kgvepitwee mmhdlglkde

RelE$_{Sp2}$ (pHi=11.1):
mnnlyklvpt rrfikqlkkl drytqklitn ylqtnvledp rrhgkalvgn
rvgqwryrig nyrvivqivd delvvatlev ghrrdiy

Fig. 10

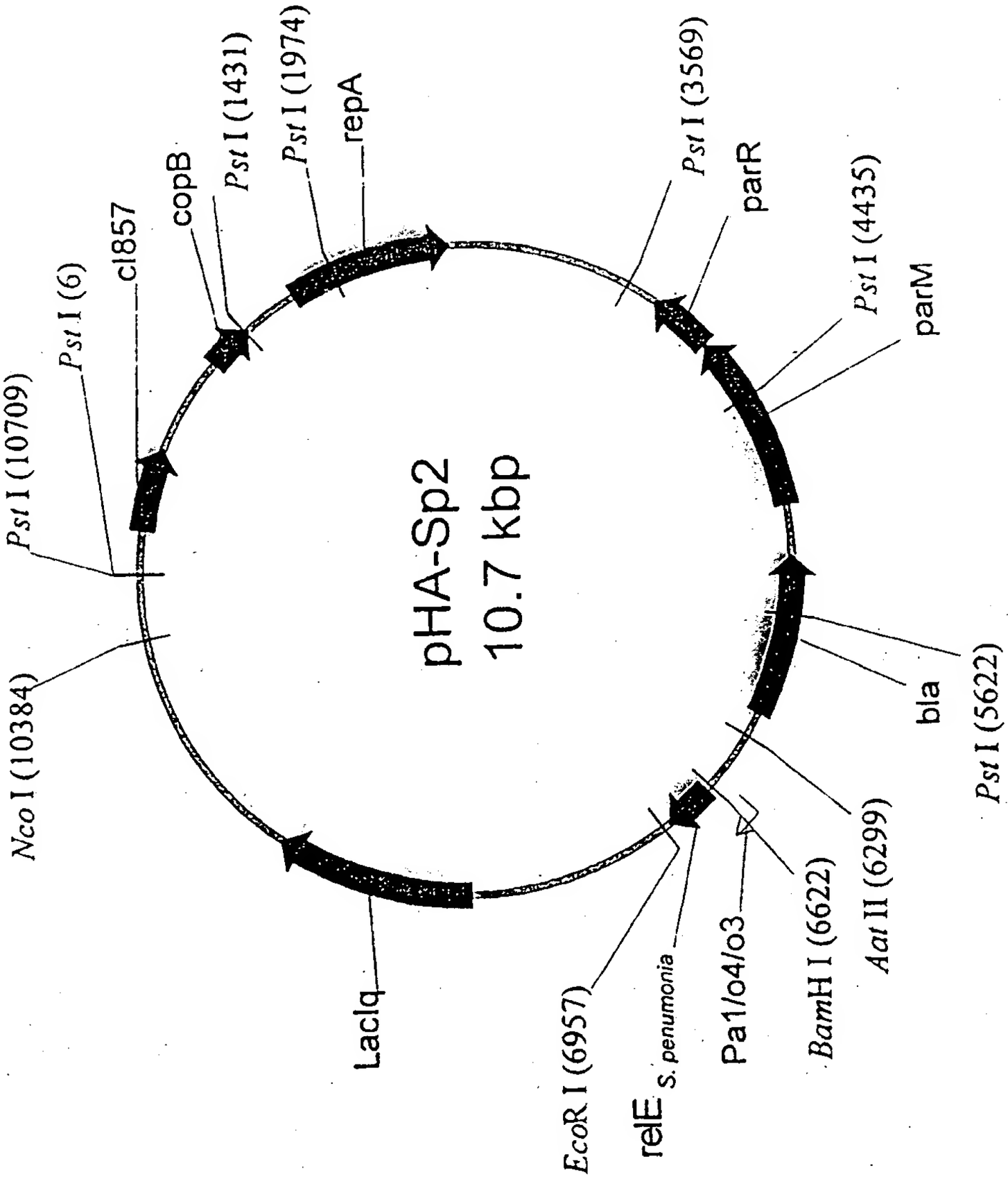
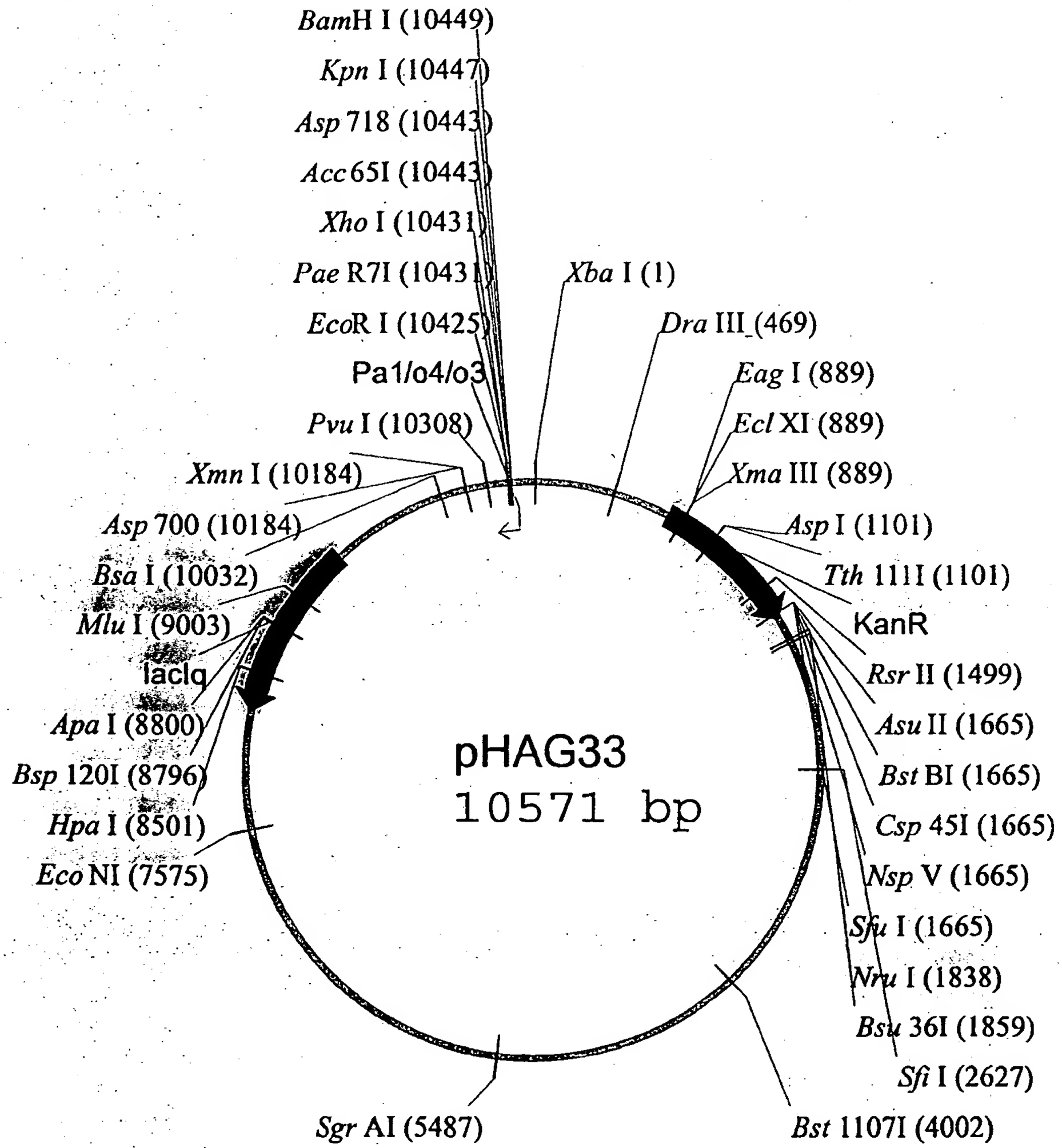

pHA-Sp2
10.7 kbp
PstI (10709)
PstI (6)
cI857
copB
PstI (1431)
PstI (1974)
repA
PstI (3569)
parR
PstI (4435)
parM
bla
PstI (5622)
AatII (6299)
BamHI (6622)
Pa1/o4/o3
EcoRI (6957)
relE S. penumonia
LacIq
NcoI (10384)

Fig. 11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Kpn I (10447)
Asp 718 (10443)
Acc 65I (10443)
Xho I (10431)
Pae R7I (10431)
EcoR I (10425)
Pa1/o4/o3
Pvu I (10308)
Xmn I (10184)
Asp 700 (10184)
Bsa I (10032)
Mlu I (9003)
laclq
Apa I (8800)
Bsp 120I (8796)
Hpa I (8501)
Eco NI (7575)
Sgr AI (5487)
Xba I (1)
Dra III (469)
Eag I (889)
Ecl XI (889)
Xma III (889)
Asp I (1101)
Tth 111I (1101)
KanR
Rsr II (1499)
Asu II (1665)
Bst BI (1665)
Csp 45I (1665)
Nsp V (1665)
Sfu I (1665)
Nru I (1838)
Bsu 36I (1859)
Sfi I (2627)
Bst 1107I (4002)
pHAG33
10571 bp

Fig. 12

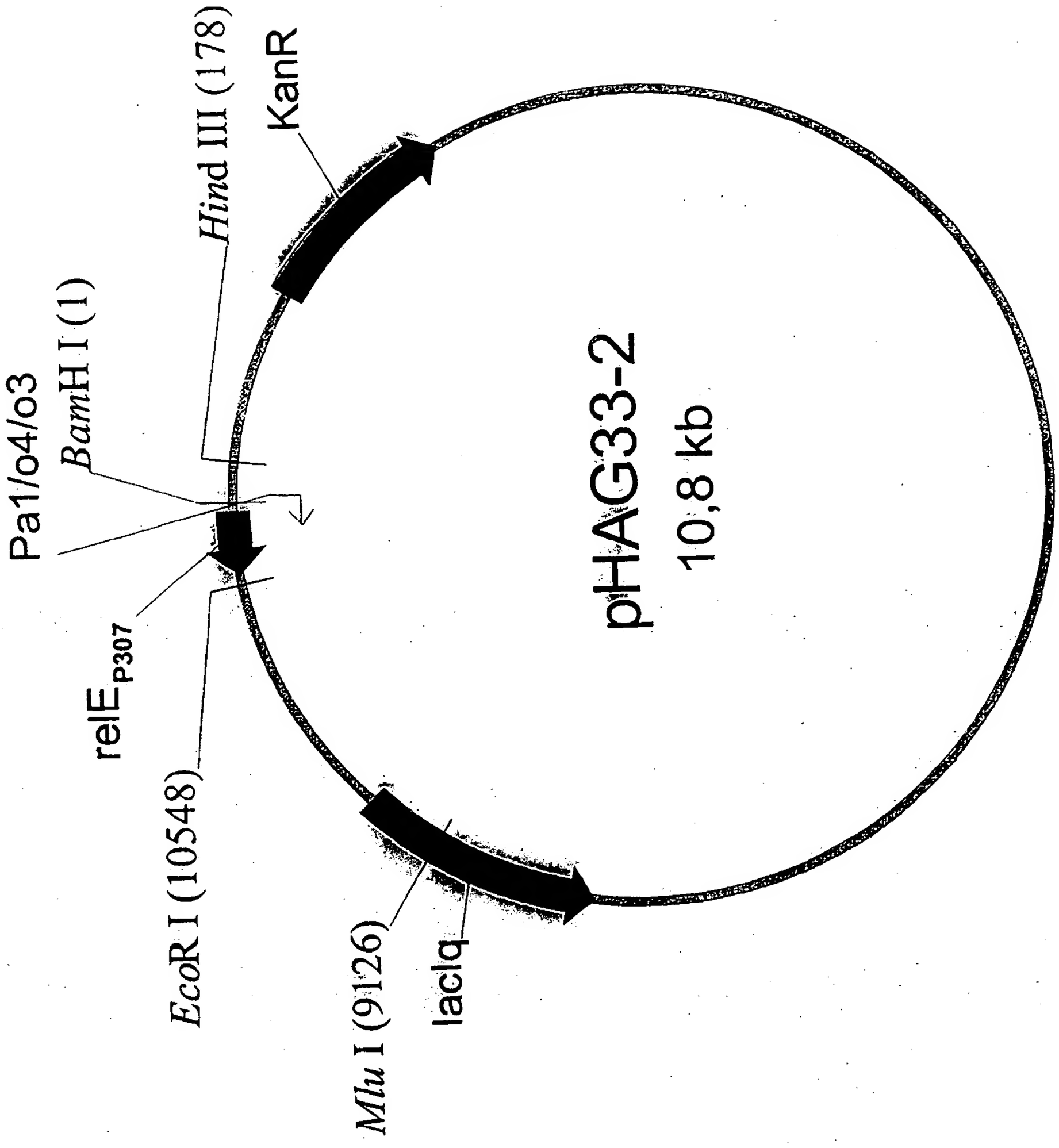
KanR
Hind III (178)
Pa1/o4/o3
BamH I (1
relE P307
EcoR I (10548)
Mlu I (9126)
laclq
pHAG33-2
10,8 kb

Fig. 13

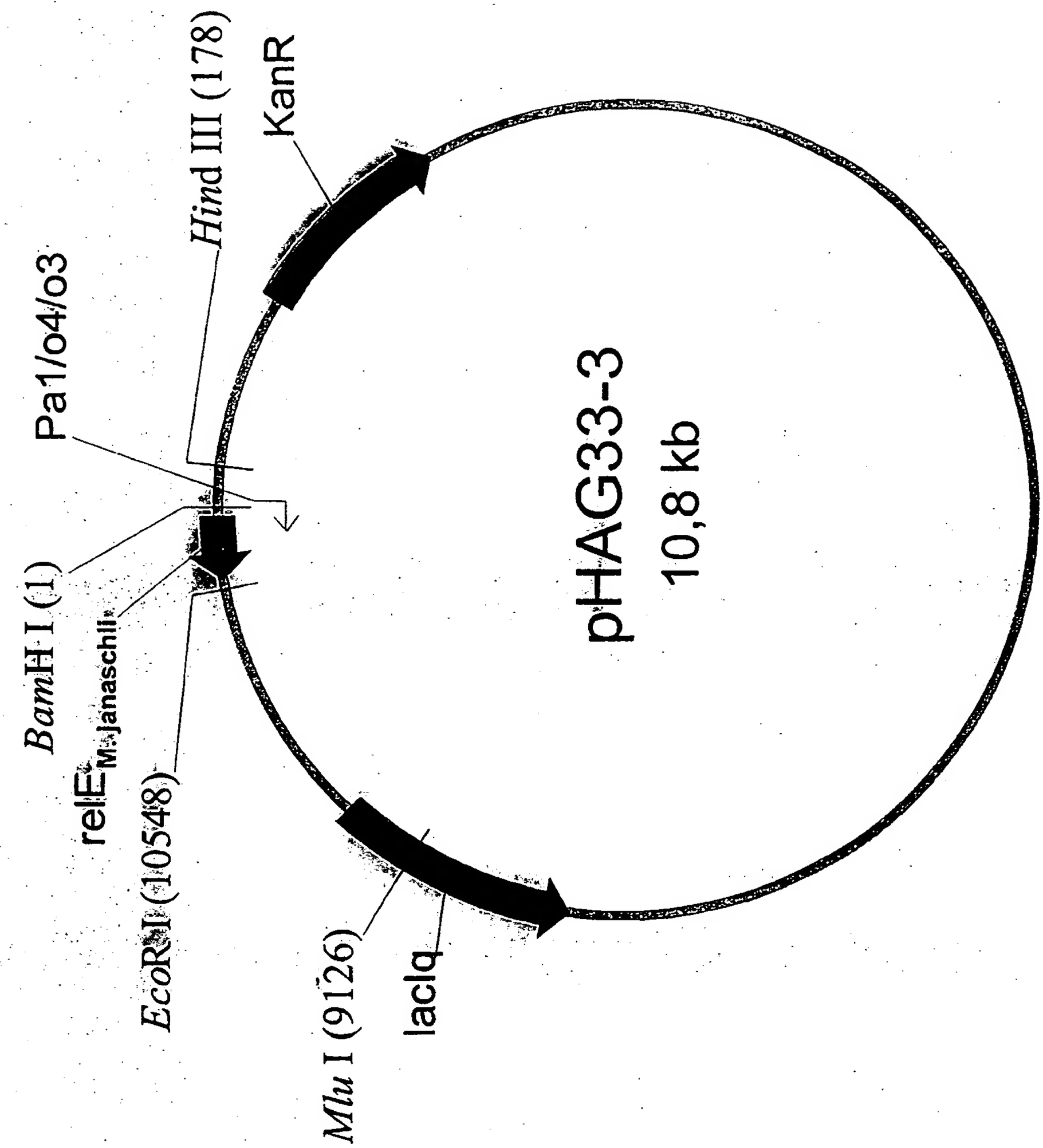
BamH I (1)
Pa1/o4/o3
relE M.janaschii
EcoR I (10548)
Hind III (178)
KanR
pHAG33-3
10,8 kb
Mlu I (9126)
lacIq

Fig. 14

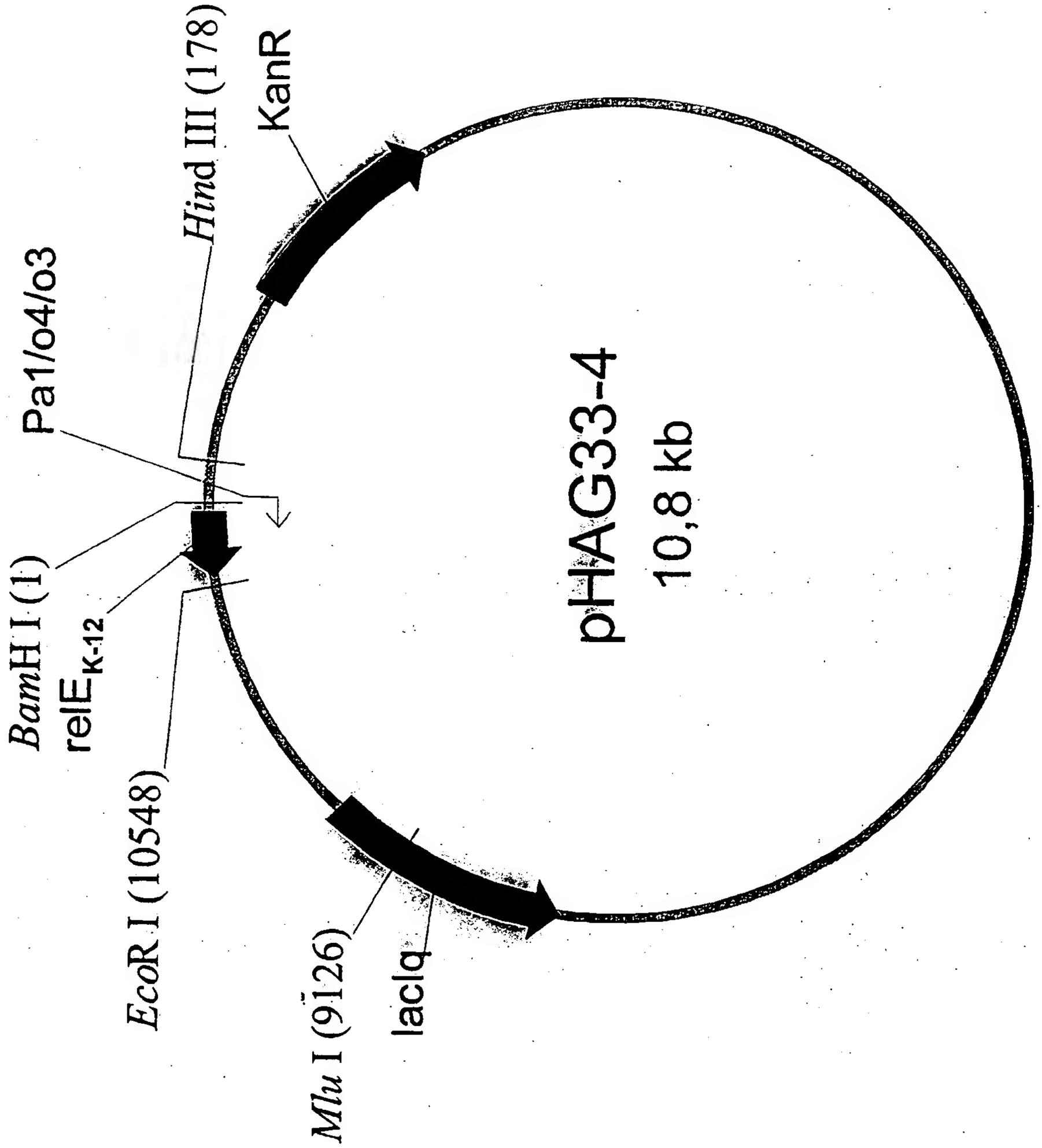
pHAG33-4
10,8 kb
BamH I (1)
relE K-12
Pa1/o4/o3
Hind III (178)
KanR
EcoR I (10548)
Mlu I (9126)
lacIq

Fig. 15

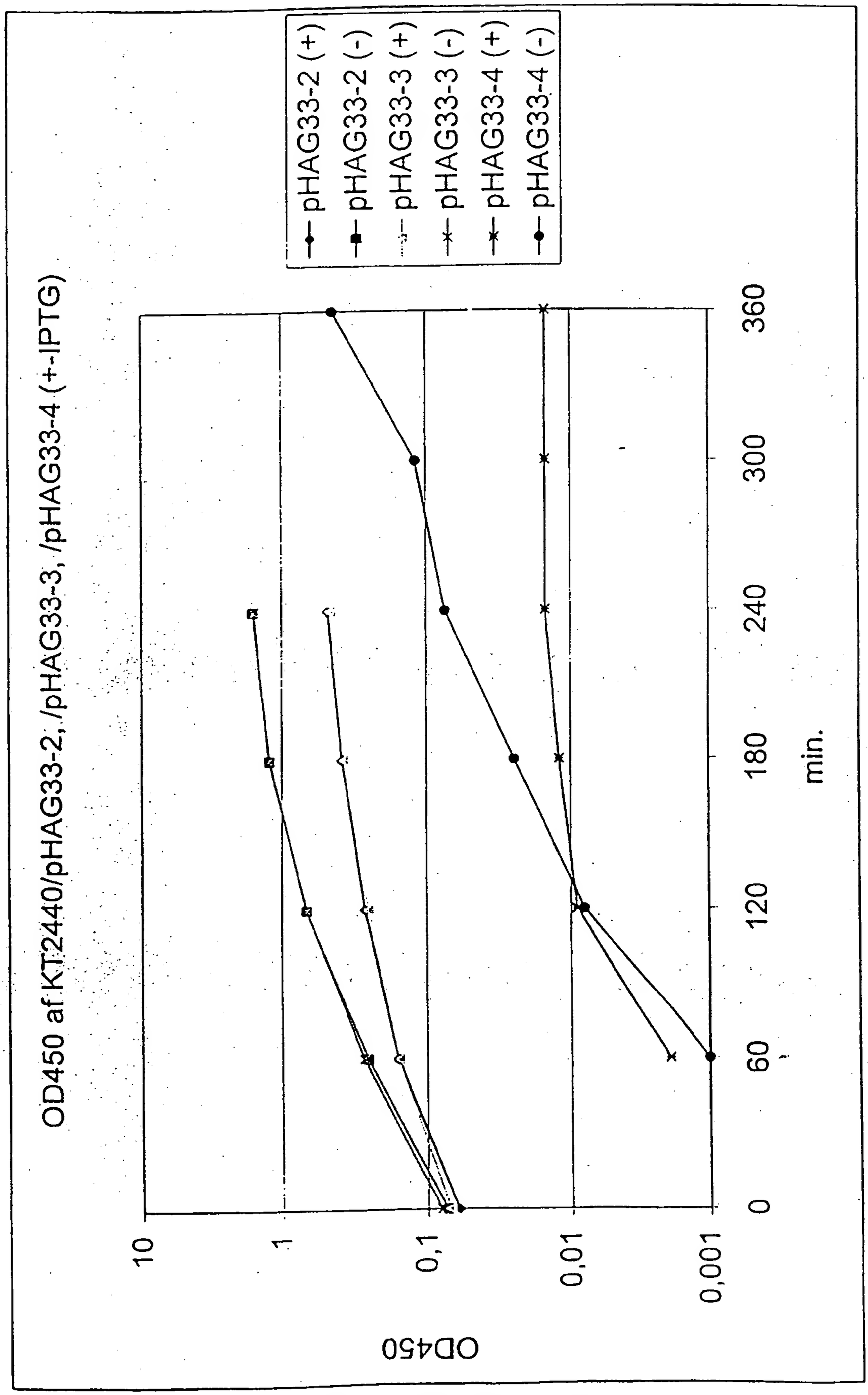
pHAG33-2 (+)
pHAG33-2 (-)
pHAG33-3 (+)
pHAG33-3 (-)
pHAG33-4 (+)
pHAG33-4 (-)
OD450 af KT2440/pHAG33-2, /pHAG33-3, /pHAG33-4 (+-IPTG)
OD450
10
1
0,1
0,01
0,001
0
60
120
180
240
300
360
min.

Fig. 16

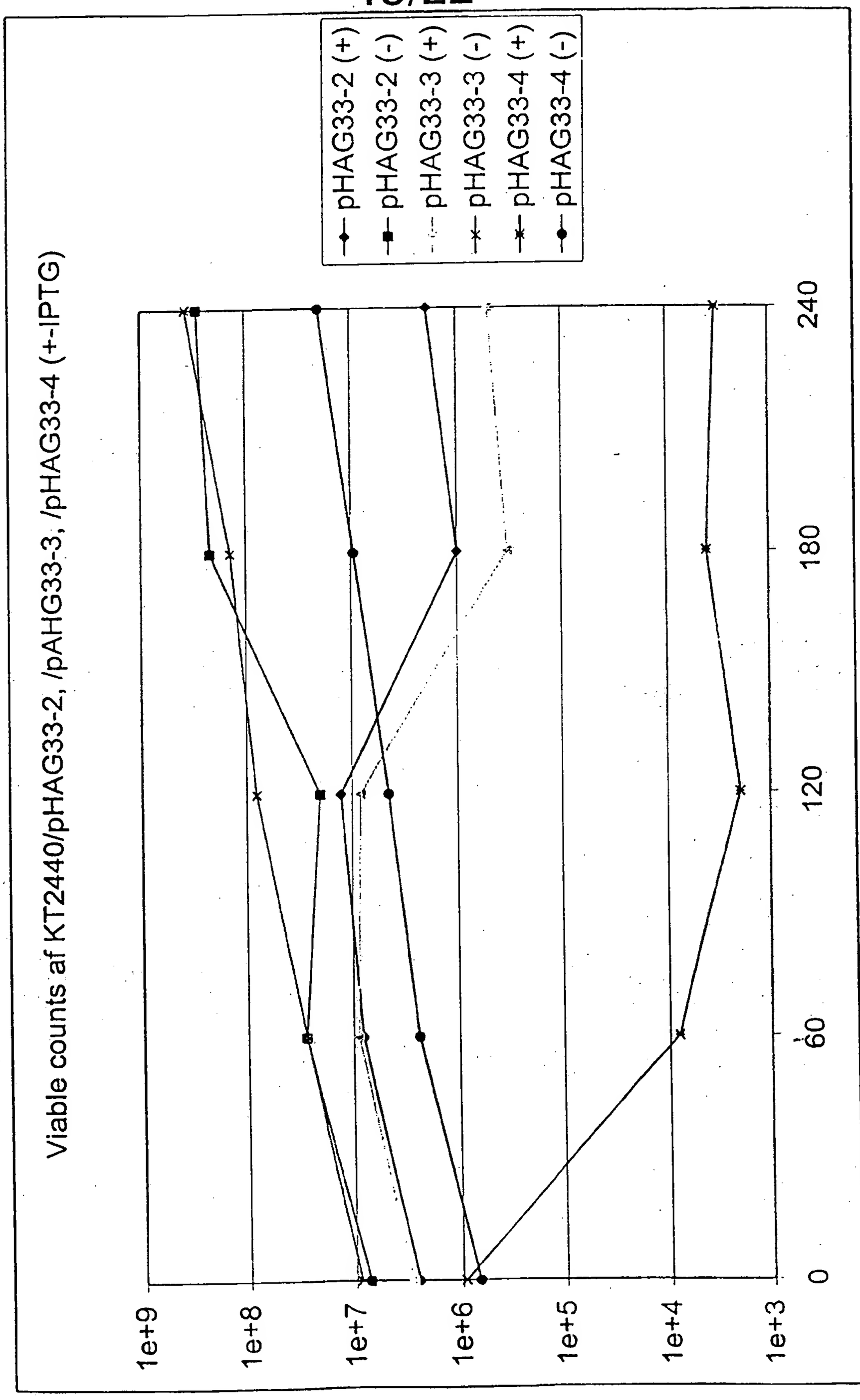
pHAG33-2 (+)
pHAG33-2 (-)
pHAG33-3 (+)
pHAG33-3 (-)
pHAG33-4 (+)
pHAG33-4 (-)
Viable counts af KT2440/pHAG33-2, /pAHG33-3, /pHAG33-4 (+-IPTG)
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
0
60
120
180
240

Fig. 17

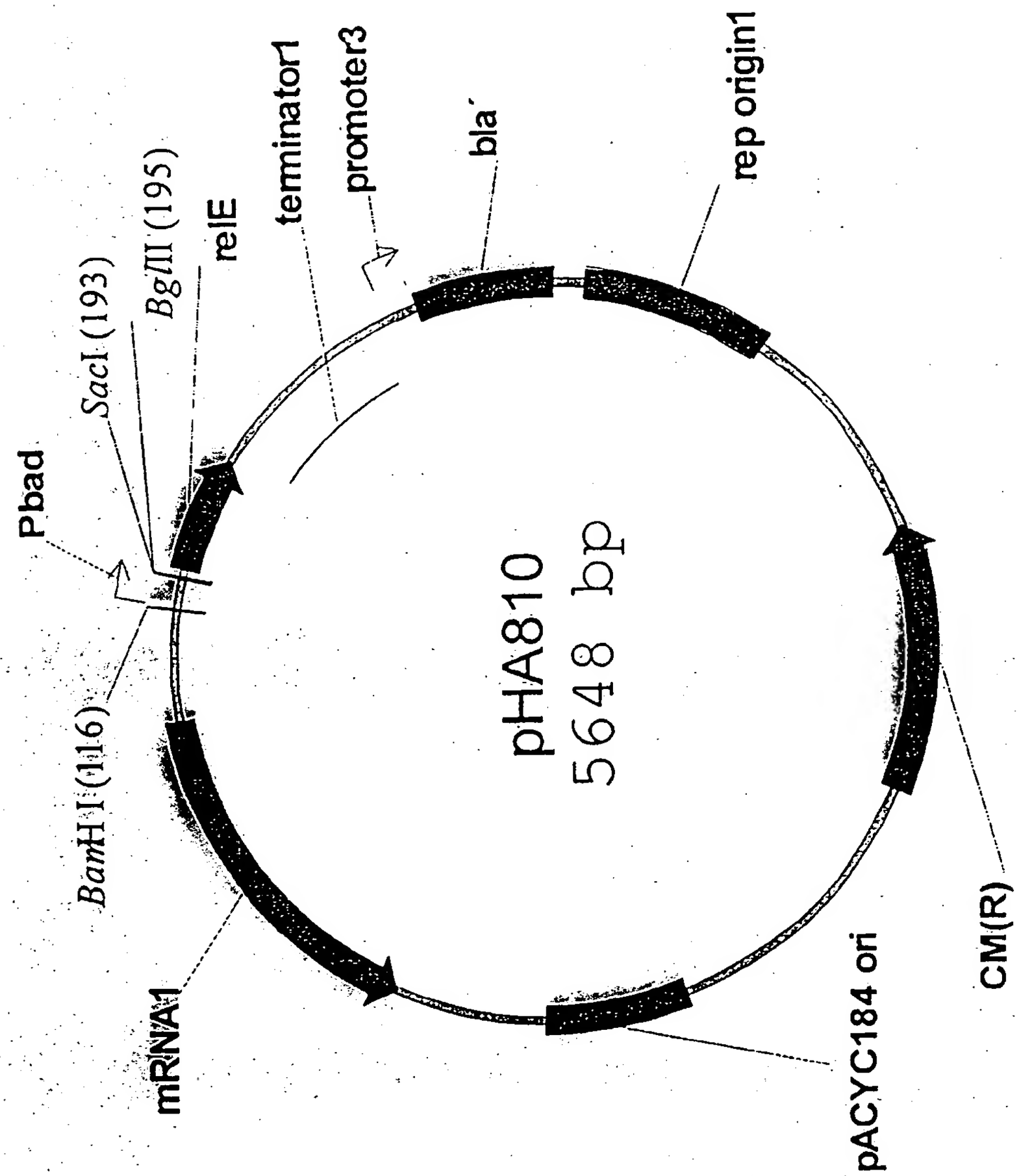
pHA810
5648 bp
Pbad
SacI (193)
BglII (1
relE
terminator1
promoter3
bla´
rep origin1
BamHI (116)
mRNA1
pACYC184 ori
CM(R)

Fig. 18

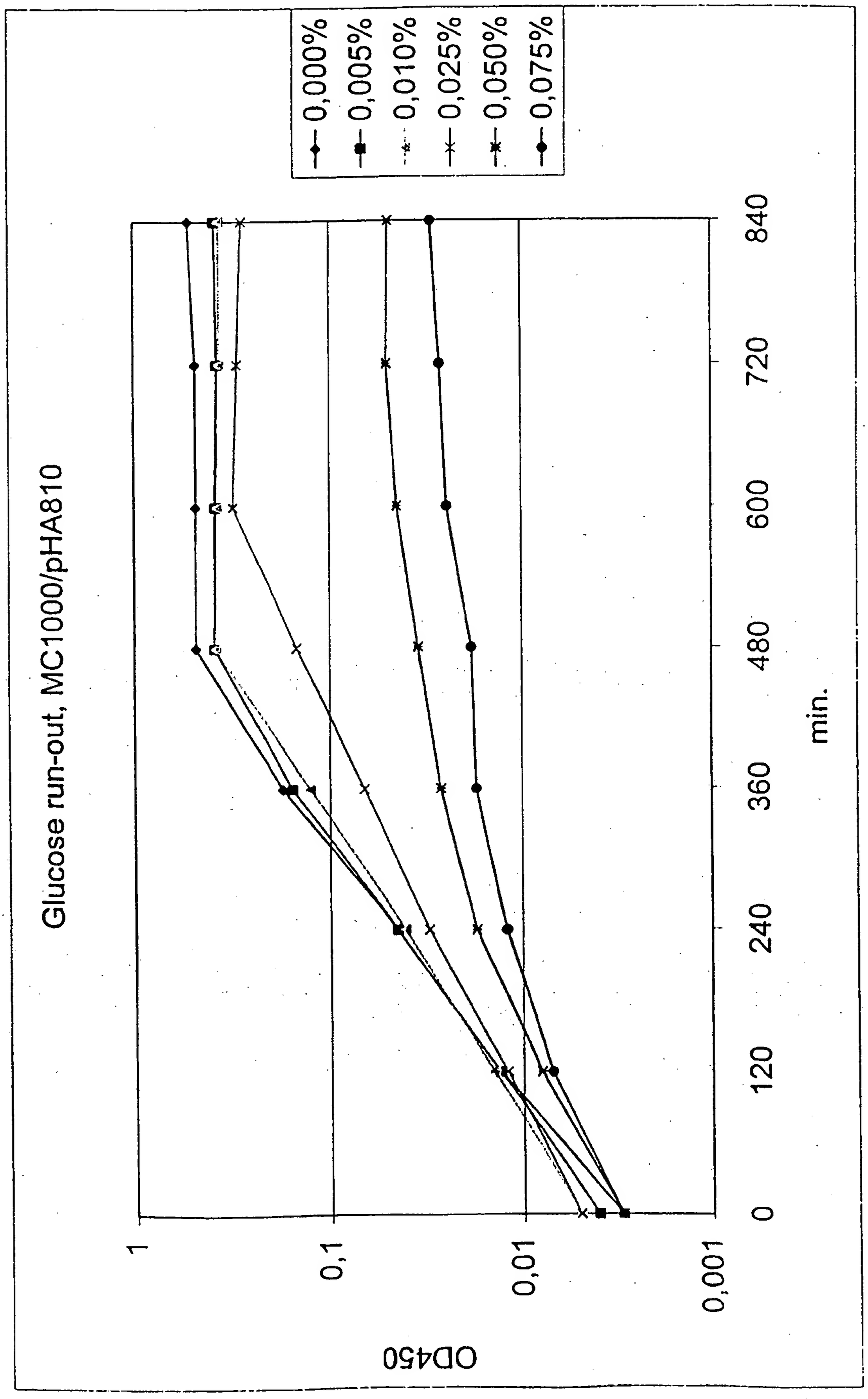
0,000%
0,005%
0,010%
0,025%
0,050%
0,075%
Glucose run-out, MC1000/pHA810
OD450
1
0,1
0,01
0,001
0
120
240
360
480
600
720
840
min.

Fig. 19

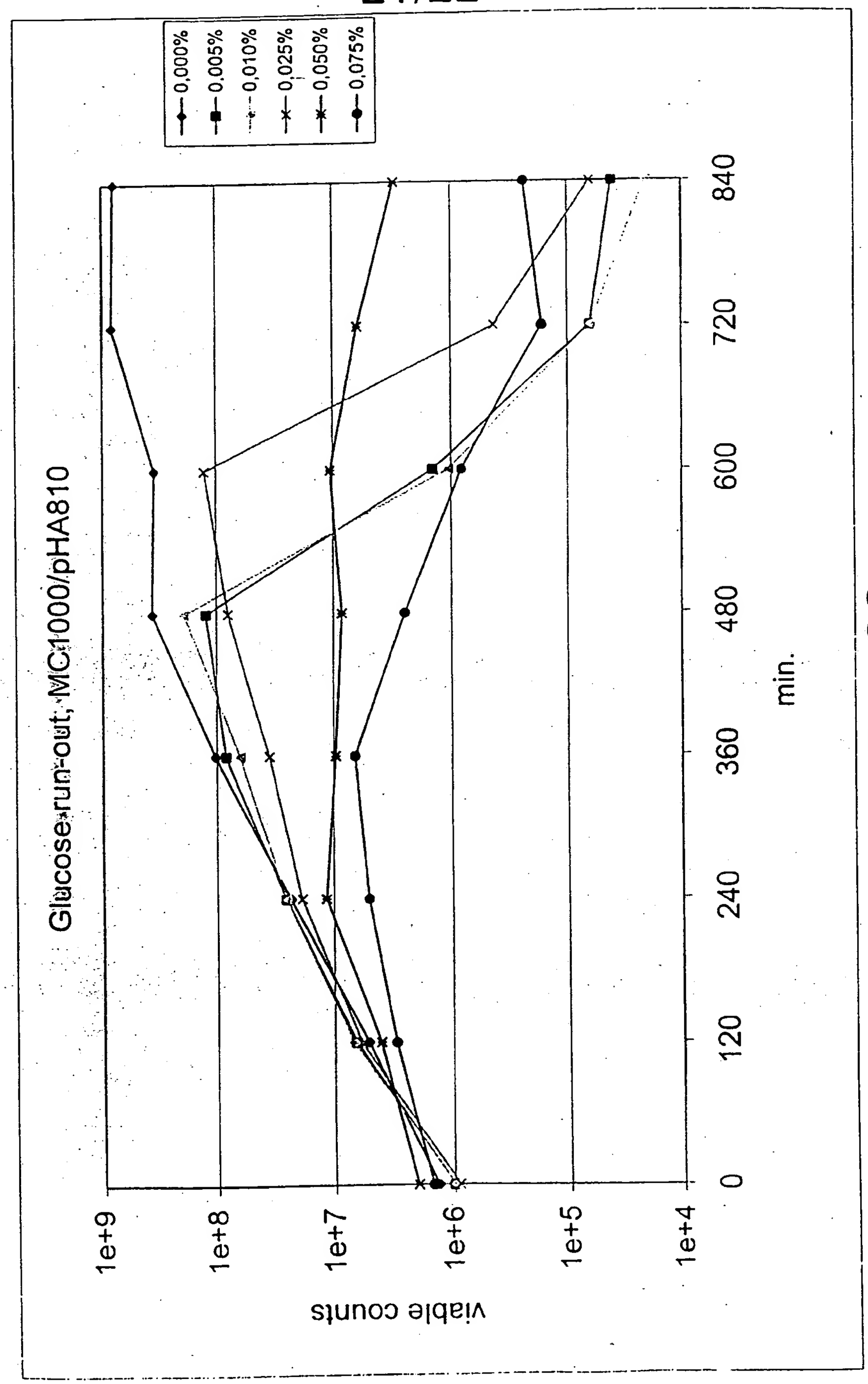
0,000%
0,005%
0,010%
0,025%
0,050%
0,075%
Glucose run-out, MC1000/pHA810
viable counts
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
0
120
240
360
480
600
720
840
min.

Fig. 20

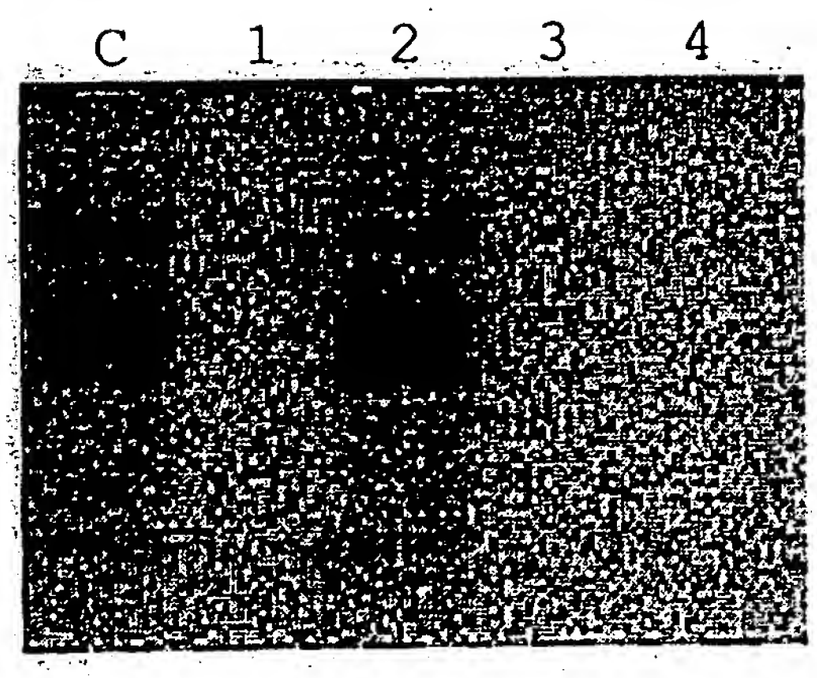

Fig. 21

SEQUENCE LISTING

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Gerdes, Kenn
Gotfredsen, Marie
Grønland, Hugo
Pedersen, Kim
Kristoffersen, Peter

<120> Cytotoxin-based biological containment

<130> 20935 PC 1

<150> PA 1998 00627
<151> 1998-05-07

<150> US 60/085,067
<151> 1998-05-12

<160> 59

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relE1B was used for the amplification of relEK-12 by PCR on pBD2430

<400> 1
ccccggatcc ataaggagtt ttataaatgg cgtattttct ggattttgac g 51

<210> 2
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relE1B was used for the amplification of relEK-12 by PCR on pBD2430

<400> 2
ccccccctcga ggtcgactca gagaatgcgt ttgaccgc 38

<210> 3
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer RelB-P307/1 was used for the generation of a PCR-fragment from pNZ945

<400> 3
ccccccggatc ccagtcttga aaggtggc 28

<210> 4
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer RelB-P307/2 was used for the generation of a PCR-fragment from pNZ945

<400> 4
cccccgaatt ctcataggta tttatccag 29

<210> 5
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relE-p307/3 was used to PCR-amplify the gene relEP307 from pNZ945

<400> 5
ccccggatcc agatctggat aaatacc 27

<210> 6
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relE-P307/2 was used to PCR-amplify the gene relEP307 from pNZ945

<400> 6
cccccgaatt cgtaactttc tgtgtttatt gc 32

<210> 7
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relE-P307/4 was used for the generation of a DNA fragment encoding relEP307 by PCR

<400> 7
cccccgagct cagatctgga taaatacc 28

<210> 8
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relE-P307/5 was used for the generation of a DNA fragment encoding relEP307 by PCR

<400> 8
ccccgcatg cgtaactttc tgtgtttatt gc 32

<210> 9
<211> 1461
<212> DNA
<213> E. coli K-12

<400> 9
cttaatttca ggccccatcg gatcacacat ggagagtttt tatgaataac cccgtctgtc 60
ttgatgactg gttgattggc tttaaaagct tgttgacagg ggtaaacgtt cggcaataat 120
tttctgccgc atgcgggtgt tgcataaaac gtgttacgtt cctttatcga caggtcaggt 180
caccgctcac ccgccgacga gaaagcaaca ctgacatgct aaagcaaaaa atagatgaat 240
aagttgagtt gtgcatatgt agcctgaccg tcacaaagta tatggtgtct gtaccagtaa 300
gatgatggcc ggactcttta aaaacgagct gacctgcaca atacaggatg gacttagcaa 360
tggctgctcc tggcacaaag cggacagtga tcaccgttct tacgactact ttctgacttc 420
cttcgtgact tgccctaagc atgttgtagt rbmrnarbst artgcgatac ttgtaatgac 480
atttgtaatt acaagaggtg taagacatgg gtargcatta acctgcgtat tgacgatgaa 540
cttaaagcgc gttcttacgc cgcgcttgaa aaaatgggtg taactccttc tgaagcgctt 600
cgtctcatgc tcgagtatat cgctgacaat gaacgcttgc cgttcaaaca gacactcctg 660
agtgatgaag atgctgaact tgtggagata gtgaaagaac ggcttcgtaa tcctndrbst 720
artraagcca gtacgtgtga cgctggatga actctgatgg cgtattttct ggattttgac 780
gagcgggcac taaaggaatg gcgaaagctg ggctcgacgg tacgtgaaca gttgaaaaag 840
aagctggttg aagtacttga gtcaccccgg attgaagcaa acaagctccg tggtatgcct 900
gattgttaca agattaagct ccggtcttca ggctatcgcc ttgtatacca ggttatagac 960
gagaaagttg tcgttttcgt gatttctgtt gggaaaagag aacgctcgga agtatatagc 1020
gaggndrcgg tcaaacgcat tctctgaacc aaagcatgac atctctgttt cgcaccgsta 1080
rthkcraagg tgacacttct gctttgcgtt gacaggagaa gcaggctatg aagcagcaaa 1140
aggcgatgtt aatcgccctg atcgtcatct gtttaaccgt catagtgacg gcactggtaa 1200
cgaggaaaga cctctgcgag gtacgaatcc gaaccgndhk caccagacgg aggtcgctgt 1260
cttcacagct tacgaacctg aggagtaaga gacccggcgg gggagaaatc cctcgccacc 1320
tctgatgtgg caggcatcct caacgcaccc gcacttaacc cgcttcggcg ggtttttgtt 1380
tttattttca arttcgcgtt tgaagttctg gacggtgccg gaatagaatc aaaaatactt 1440
aagtdataba saccsnnumb r 1461

<210> 10
<211> 88
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Mj2

<400> 10
Met Lys Val Leu Phe Ala Lys Thr Phe Val Lys Asp Leu Lys His Val
1 5 10 15
Pro Gly His Ile Arg Lys Arg Ile Lys Leu Ile Ile Glu Glu Cys Gln
20 25 30
Asn Ser Asn Ser Leu Asn Asp Leu Lys Leu Asp Ile Lys Lys Ile Lys
35 40 45
Gly Tyr His Asn Tyr Tyr Arg Ile Arg Val Gly Asn Tyr Arg Ile Gly
50 55 60
Ile Glu Val Asn Gly Asp Thr Ile Ile Phe Arg Arg Val Leu His Arg
65 70 75 80
Lys Ser Ile Tyr Asp Tyr Phe Pro
85

<210> 11
<211> 91
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Mj3

<400> 11
Met Lys Gln Trp Lys Tyr Leu Leu Lys Lys Ser Phe Ile Lys Asp Leu
1 5 10 15
Lys Glu Leu Pro Lys Asn Ile Gln Glu Lys Ile Lys Lys Leu Val Phe
20 25 30
Glu Glu Ile Pro Asn Lys Asn Asn Pro Pro Glu Ile Pro Asn Val Lys
35 40 45
Lys Leu Lys Gly Ala Asp Ser Tyr Tyr Arg Ile Arg Val Gly Asp Tyr
50 55 60
Arg Ile Gly Phe Lys Tyr Glu Asn Gly Lys Ile Val Phe Tyr Arg Val
65 70 75 80
Leu His Arg Lys Gln Ile Tyr Lys Arg Phe Pro
85 90

<210> 12
<211> 87
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Af1

<400> 12
Met Phe Arg Val Val Val His Arg Lys Ala Thr Gln Glu Leu Lys Arg
1 5 10 15
Leu Lys Lys Ala His Leu Lys Lys Phe Gly Val Leu Leu Glu Thr Leu
20 25 30
Lys Thr Asp Pro Ile Pro Trp Lys Arg Phe Asp Val Lys Lys Ile Glu
35 40 45
Gly Glu Glu Asn Thr Tyr Arg Ile Arg Ile Gly Asp Phe Arg Val Ile
50 55 60
Tyr Phe Leu Asp Lys Pro Thr Lys Thr Val His Ile Leu Lys Val Glu
65 70 75 80
Arg Arg Gly Lys Val Tyr Asp
85

<210> 13
<211> 90
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Mj1

<400> 13
Met Lys Phe Asn Val Glu Ile His Lys Arg Val Leu Lys Asp Leu Lys
1 5 10 15
Asp Leu Pro Pro Ser Asn Leu Lys Lys Phe Lys Glu Leu Ile Glu Thr
20 25 30
Leu Lys Thr Asn Pro Ile Pro Lys Glu Lys Phe Asp Ile Lys Arg Leu
35 40 45

Lys Gly Ser Asp Glu Val Tyr Arg Val Arg Ile Gly Lys Phe Arg Val
50 55 60
Gln Tyr Val Val Leu Trp Asp Asp Arg Ile Ile Ile Ile Arg Lys Ile
65 70 75 80
Ser Arg Arg Glu Gly Ala Tyr Lys Asn Pro
85 90

<210> 14
<211> 74
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Bt

<400> 14
Met Lys Phe Ile Ala Lys Gln Glu Lys Gly Ile Gln Lys Arg Ile Ala
1 5 10 15
Glu Gly Leu Lys Gly Leu Leu Lys Ile Pro Pro Glu Gly Asp Ile Lys
20 25 30
Ser Met Lys Gly Tyr Thr Glu Leu Tyr Arg Leu Arg Ile Gly Thr Phe
35 40 45
Arg Ile Leu Phe Glu Ile Asn His Asp Glu Lys Val Ile Tyr Ile Gln
50 55 60
Ala Ile Gly Asn Arg Gly Asp Ile Tyr Lys
65 70

<210> 15
<211> 95
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-P307

<400> 15
Met Arg Tyr Gln Val Lys Phe Arg Glu Asp Ala Leu Lys Glu Trp Gln
1 5 10 15
Lys Leu Asp Lys Ala Ile Gln Gln Gln Phe Ala Lys Lys Leu Lys Lys
20 25 30
Cys Cys Asp Asn Pro His Ile Pro Ser Ala Lys Leu Arg Gly Ile Lys
35 40 45
Asp Cys Tyr Lys Ile Lys Leu Arg Ala Ser Gly Phe Arg Leu Val Tyr
50 55 60
Gln Val Ile Asp Glu Gln Leu Ile Ile Ala Val Val Ala Val Gly Lys
65 70 75 80
Arg Glu Arg Ser Asp Val Tyr Asn Leu Ala Ser Glu Arg Met Arg
85 90 95

<210> 16
<211> 95
<212> PRT
<213> Bacterium

<220>
<223> protein RelEK-12

<400> 16
Met Ala Tyr Phe Leu Asp Phe Asp Glu Arg Ala Leu Lys Glu Trp Arg
1 5 10 15
Lys Leu Gly Ser Thr Val Arg Glu Gln Leu Lys Lys Lys Leu Val Glu
20 25 30
Val Leu Glu Ser Pro Arg Ile Glu Ala Asn Lys Leu Arg Gly Met Pro
35 40 45
Asp Cys Tyr Lys Ile Lys Leu Arg Ser Ser Gly Tyr Arg Leu Val Tyr
50 55 60
Gln Val Ile Asp Glu Lys Val Val Val Phe Val Ile Ser Val Gly Lys
65 70 75 80
Arg Glu Arg Ser Glu Val Tyr Ser Glu Ala Val Lys Arg Ile Leu
85 90 95

<210> 17
<211> 96
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Vc

<400> 17
Met Thr Tyr Lys Leu Glu Phe Lys Lys Ser Ala Leu Lys Glu Trp Lys
1 5 10 15
Lys Leu Ala Val Pro Leu Gln Gln Gln Phe Lys Lys Lys Leu Ile Glu
20 25 30
Arg Leu Glu Asn Pro His Val Pro Ser Ala Lys Leu Ser Gly Ala Glu
35 40 45
Asn Ile Tyr Lys Ile Lys Leu Arg Gln Ser Gly Tyr Arg Leu Val Tyr
50 55 60
Gln Val Glu Asn Asp Ile Ile Val Val Thr Val Leu Ala Val Gly Lys
65 70 75 80
Arg Glu Arg Ser Glu Val Tyr Thr Lys Ala Leu Gln Arg Leu Asp Asp
85 90 95

<210> 18
<211> 87
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Mt1

<400> 18
Met Pro Tyr Thr Val Arg Phe Thr Thr Thr Ala Arg Arg Asp Leu His
1 5 10 15
Lys Leu Pro Pro Arg Ile Leu Ala Ala Val Val Glu Phe Ala Phe Gly
20 25 30
Asp Leu Ser Arg Glu Pro Leu Arg Val Gly Lys Pro Leu Arg Arg Glu
35 40 45
Leu Ala Gly Thr Phe Ser Ala Arg Arg Gly Thr Tyr Arg Leu Leu Tyr
50 55 60
Arg Ile Asp Asp Glu His Thr Thr Val Val Ile Leu Arg Val Asp His
65 70 75 80
Arg Ala Asp Ile Tyr Arg Arg
85

<210> 19
<211> 97
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Mt2

<400> 19
Met Ser Asp Asp His Pro Tyr His Val Ala Ile Thr Ala Thr Ala Ala
1 5 10 15
Arg Asp Leu Gln Arg Leu Pro Glu Lys Ile Ala Ala Ala Cys Val Glu
20 25 30
Phe Val Phe Gly Pro Leu Leu Asn Asn Pro His Arg Leu Gly Lys Pro
35 40 45
Leu Arg Asn Asp Leu Glu Gly Leu His Ser Ala Arg Arg Gly Asp Tyr
50 55 60
Arg Val Val Tyr Ala Ile Asp Asp Gly His His Arg Val Glu Ile Ile
65 70 75 80
His Ile Ala Arg Arg Ser Ala Ser Tyr Arg Met Asn Pro Cys Arg Pro
85 90 95
Arg

<210> 20
<211> 102
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Hi

<400> 20
Met Ser Glu Glu Lys Pro Leu Lys Val Ser Tyr Ser Lys Gln Phe Val
1 5 10 15
Arg Asp Leu Thr Asp Leu Ala Lys Arg Ser Pro Asn Val Leu Ile Gly
20 25 30
Ser Lys Tyr Ile Thr Ala Ile His Cys Leu Leu Asn Arg Leu Pro Leu
35 40 45
Pro Glu Asn Tyr Gln Asp His Ala Leu Val Gly Glu Trp Lys Gly Tyr
50 55 60
Arg Asp Cys His Ile Gln Gly Asp Leu Val Leu Ile Tyr Gln Tyr Val
65 70 75 80
Ile Gln Asp Glu Phe Asp Glu Leu Lys Phe Ser Arg Leu Asn Ile His
85 90 95
Ser Gln Thr Ala Leu Lys
100

<210> 21
<211> 92
<212> PRT
<213> Bacterium

<220>
<223> protein RelEk12sos

<400> 21
Met Ile Gln Arg Asp Ile Glu Tyr Ser Gly Gln Tyr Ser Lys Asp Val
1 5 10 15
Lys Leu Ala Gln Lys Arg His Lys Asp Met Asn Lys Leu Lys Tyr Leu
20 25 30
Met Thr Leu Leu Ile Asn Asn Thr Leu Pro Leu Pro Ala Val Tyr Lys
35 40 45
Asp His Pro Leu Gln Gly Ser Trp Lys Gly Tyr Arg Asp Ala His Val
50 55 60
Glu Pro Asp Trp Ile Leu Ile Tyr Lys Leu Thr Asp Lys Leu Leu Arg
65 70 75 80
Phe Glu Arg Thr Gly Thr His Ala Ala Leu Phe Gly
85 90

<210> 22
<211> 88
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Hp

<400> 22
Met Leu Lys Leu Asn Leu Lys Lys Ser Phe Gln Lys Asp Phe Asp Lys
1 5 10 15
Leu Leu Leu Asn Gly Phe Asp Asp Ser Val Leu Asn Glu Val Ile Leu
20 25 30
Thr Leu Arg Lys Lys Glu Pro Leu Asp Pro Gln Phe Gln Asp His Ala
35 40 45
Leu Lys Gly Lys Trp Lys Pro Tyr Arg Glu Cys His Ile Lys Pro Asp
50 55 60
Val Leu Leu Val Tyr Leu Val Lys Asp Asp Glu Leu Ile Leu Leu Arg
65 70 75 80
Leu Gly Ser His Ser Glu Leu Phe
85

<210> 23
<211> 92
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Af2

<400> 23
Met Ala Trp Lys Val Arg Tyr His Lys Lys Ala Ile Lys Phe Leu Glu
1 5 10 15
Lys Leu Asp Glu Gly Lys Arg Ser Ile Leu Leu Ser Lys Ile Gln Glu
20 25 30
Leu Val Asn Ser Leu Glu Ser Gly Val Leu Pro Ile Gln Arg Met Asp
35 40 45
Ile Lys Arg Leu Lys Gly Val Trp Asp Gly Phe Leu Arg Leu Arg Val
50 55 60
Gly Glu Val Arg Ile Ile Phe Lys Ile Asn Val Glu Asp Glu Thr Ile
65 70 75 80
Phe Ile Tyr Ser Ile His Phe Arg Glu Lys Val Tyr
85 90

<210> 24
<211> 86
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Af4

<400> 24
Met Asn Glu Val Leu Ile His Lys Lys Phe Leu Asp Gly Leu Asp Ser
1 5 10 15
Gly Arg Arg Ser Lys Val Leu Asp Ala Ile Arg Met Leu Lys Asp Phe
20 25 30
Pro Ile Ile Arg Ala Asp Ile Lys Lys Ile Gly Pro Lys Thr Tyr Arg
35 40 45
Leu Arg Lys Gly Glu Ile Arg Ile Ile Phe Asp Phe Asp Ile Gly Thr
50 55 60
Asn Arg Val Phe Val Lys Phe Ala Ala Ser Glu Gly Val Phe Thr Lys
65 70 75 80
Thr Glu Glu Lys Phe Phe
85

<210> 25
<211> 85
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Af3

<400> 25
Met Asn Tyr Lys Ala Gln Phe Ser Glu Glu Phe Leu Lys Ile Ala Lys
1 5 10 15
Lys Leu Lys Glu Lys Asp Pro Glu Leu Leu Lys Arg Leu Gln Ser Lys
20 25 30
Val Glu Glu Ile Ile Lys Gln Pro Glu His Tyr Lys Pro Leu Arg Gly
35 40 45
Gln Met Lys Gly Leu Arg Arg Ala His Val Gly Lys Phe Val Ile Ile
50 55 60
Phe Lys Val Glu Glu Asp Thr Val Lys Phe Val Thr Phe Lys His His
65 70 75 80
Asn His Ala Tyr Lys
85

<210> 26
<211> 120
<212> PRT
<213> Bacterium

<220>
<223> protein RelE-Sy

<400> 26
Met Ser Asn Asn Leu His Leu Val Asn Ile Asp Phe Thr Pro Glu Tyr
1 5 10 15
Arg Arg Ser Leu Lys Tyr Leu Ala Lys Lys Tyr Arg Asn Ile Arg Ser
20 25 30
Asp Val Gln Pro Ile Ile Glu Ala Leu Gln Lys Gly Val Ile Ser Gly
35 40 45

Asp Arg Leu Ala Gly Phe Gly Ser Asp Ile Tyr Val Tyr Lys Leu Arg
50 55 60
Ile Lys Asn Ser Asn Ile Gln Lys Gly Lys Ser Ser Gly Tyr Arg Leu
65 70 75 80
Ile Tyr Leu Leu Glu Ser Glu Asn Ser Ile Leu Leu Leu Thr Ile Tyr
85 90 95
Ser Lys Ala Glu Gln Glu Asp Ile Ala Ala Ser Asp Ile Asn Ser Ile
100 105 110
Leu Gly Glu Tyr Ser Ile Glu Asp
115 120

<210> 27
<211> 86
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-SOS

<400> 27
Met Ala Ala Asn Ala Phe Val Arg Ala Arg Ile Asp Glu Asp Leu Lys
1 5 10 15
Asn Gln Ala Ala Asp Val Leu Ala Gly Met Gly Leu Thr Ile Ser Asp
20 25 30
Leu Val Arg Ile Thr Leu Thr Lys Val Ala Arg Glu Lys Ala Leu Pro
35 40 45
Phe Asp Leu Arg Glu Pro Asn Gln Leu Thr Ile Gln Ser Ile Lys Asn
50 55 60
Ser Glu Ala Gly Ile Asp Val His Lys Ala Lys Asp Ala Asp Asp Leu
65 70 75 80
Phe Asp Lys Leu Gly Ile
85

<210> 28
<211> 82
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-VC

<400> 28
Met Thr Thr Arg Ile Leu Ala Asp Val Ala Ala Ser Ile Thr Glu Phe
1 5 10 15
Lys Ala Asn Pro Met Lys Val Ala Thr Ser Ala Phe Gly Ala Pro Val
20 25 30
Ala Val Leu Asn Arg Asn Glu Pro Ala Phe Tyr Cys Val Pro Ala Ser
35 40 45
Thr Tyr Glu Ile Met Met Asp Lys Leu Glu Asp Leu Glu Leu Leu Ala
50 55 60
Ile Ala Lys Glu Arg Leu Ser Glu Asp Ser Val Ser Val Asn Ile Asp
65 70 75 80
Asp Leu

<210> 29
<211> 83
<212> PRT
<213> Bacterium

<220>
<223> protein RelB

<400> 29
Met Pro Asn Ile Ile Leu Ser Asp Thr Ser Ala Ser Val Ser Glu Leu
1 5 10 15
Lys Lys Asn Pro Met Ala Thr Val Ser Ala Gly Asp Gly Phe Pro Val
20 25 30
Ala Ile Leu Asn Arg Asn Gln Pro Ala Phe Tyr Cys Val Pro Ala Glu
35 40 45
Leu Tyr Glu Lys Met Leu Asp Ala Leu Asp Asp Gln Glu Leu Val Lys
50 55 60
Leu Val Ala Glu Arg Ser Asn Gln Pro Leu His Asp Val Asp Leu Asp
65 70 75 80
Lys Tyr Leu

<210> 30
<211> 93
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-Mt1

<400> 30
Met Arg Ile Leu Pro Ile Ser Thr Ile Lys Gly Lys Leu Asn Glu Phe
1 5 10 15
Val Asp Ala Val Ser Ser Thr Gln Asp Gln Ile Thr Ile Thr Lys Asn
20 25 30
Gly Ala Pro Ala Ala Val Leu Val Gly Ala Asp Glu Trp Glu Ser Leu
35 40 45
Gln Glu Thr Leu Tyr Trp Leu Ala Gln Pro Gly Ile Arg Glu Ser Ile
50 55 60
Ala Glu Ala Asp Ala Asp Ile Ala Ser Gly Arg Thr Tyr Gly Glu Asp
65 70 75 80
Glu Ile Arg Ala Glu Phe Gly Val Pro Arg Arg Pro His
85 90

<210> 31
<211> 89
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-Mt2

<400> 31
Met Ala Val Val Pro Leu Gly Glu Val Arg Asn Arg Leu Ser Glu Tyr
1 5 10 15
Val Ala Glu Val Glu Leu Thr His Glu Arg Ile Thr Ile Thr Arg His
20 25 30
Gly His Pro Ala Ala Val Leu Ile Ser Ala Asp Asp Leu Ala Ser Ile
35 40 45

Glu Glu Thr Leu Glu Val Leu Arg Thr Pro Gly Ala Ser Glu Ala Ile
50 55 60
Arg Glu Gly Leu Ala Asp Val Ala Ala Gly Arg Phe Val Ser Asn Asp
65 70 75 80
Glu Ile Arg Asn Arg Tyr Thr Ala Arg
85

<210> 32
<211> 97
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-K12-2

<400> 32
Met His Arg Ile Leu Ala Glu Lys Ser Val Asn Ile Thr Glu Leu Arg
1 5 10 15
Lys Asn Pro Ala Lys Tyr Phe Ile Asp Gln Pro Val Ala Val Leu Ser
20 25 30
Asn Asn Arg Pro Ala Gly Tyr Leu Leu Ser Ala Ser Ala Phe Glu Ala
35 40 45
Leu Met Asp Met Leu Ala Glu Gln Glu Glu Lys Lys Pro Ile Lys Ala
50 55 60
Arg Phe Arg Pro Ser Ala Ala Arg Leu Glu Glu Ile Thr Arg Arg Ala
65 70 75 80
Glu Gln Tyr Leu Asn Asp Met Thr Asp Asp Asp Phe Asn Asp Phe Lys
85 90 95
Glu

<210> 33
<211> 68
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-St

<400> 33
Met Phe Met Arg Thr Val Asn Tyr Ser Glu Ala Arg Gln Asn Leu Ala
1 5 10 15
Glu Val Leu Glu Ser Ala Val Thr Gly Gly Pro Val Thr Ile Thr Arg
20 25 30
Arg Gly His Lys Ser Ala Val Ile Ile Ser Ala Glu Glu Phe Glu Arg
35 40 45
Tyr Gln Thr Ala Arg Met Asp Asp Glu Phe Ala Ala Ile Met Ala Val
50 55 60
His Gly Asn Glu
65

<210> 34
<211> 79
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-coli

<400> 34
Met Gly Ser Ile Asn Leu Arg Ile Asp Asp Glu Leu Lys Ala Arg Ser
1 5 10 15
Tyr Ala Ala Leu Glu Lys Met Gly Val Thr Pro Ser Glu Ala Leu Arg
20 25 30
Leu Met Leu Glu Tyr Ile Ala Asp Asn Glu Arg Leu Pro Phe Lys Gln
35 40 45
Thr Leu Leu Ser Asp Glu Asp Ala Glu Leu Val Glu Ile Val Lys Glu
50 55 60
Arg Leu Arg Asn Pro Lys Pro Val Arg Val Thr Leu Asp Glu Leu
65 70 75

<210> 35
<211> 98
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-Hi

<400> 35
Met Ala Leu Thr Asn Ser Ser Ile Ser Phe Arg Thr Val Glu Lys Thr
1 5 10 15
Lys Leu Glu Ala Tyr Gln Val Ile Glu Gln Tyr Gly Leu Thr Pro Ser
20 25 30
Gln Val Phe Asn Met Phe Leu Ala Gln Ile Ala Lys Thr Arg Ser Ile
35 40 45
Pro Val Asp Leu Asn Tyr Leu Arg Pro Asn Lys Glu Thr Leu Ala Ala
50 55 60
Ile Asp Glu Leu Asp Ser Gly Asn Ala Glu Ser Phe Phe Ile Glu Ala
65 70 75 80
Ser Glu Asn Tyr Ser Ala Glu Glu Phe Thr Lys Arg Ile Leu Asn Gly
85 90 95
Gly Gln

<210> 36
<211> 82
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-MJ

<400> 36
Met Leu Asn Ile Asn Lys Glu Ile Ala Gln Ile Glu Thr Glu Leu Asn
1 5 10 15
Glu Leu Lys Lys Leu Arg Asp Glu Ile Ser Glu Arg Ile Glu Lys Leu
20 25 30
Glu Ile Lys Leu Leu Lys Leu Lys Ala Leu Ala Ile Pro Glu Glu Glu
35 40 45
Phe Glu Glu Asp Tyr Glu Glu Ile Ile Glu Asp Val Lys Lys Ser Leu
50 55 60
Asp Lys Lys Glu Thr Val Pro Ala Glu Glu Ala Leu Lys Glu Leu Gly
65 70 75 80
Leu Leu

<210> 37
<211> 65
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-Af1

<400> 37
Met Asn Glu Ala Leu Leu Arg Glu Ile Tyr Ser Glu Val Lys Lys Ile
1 5 10 15
Arg Glu Lys Ile Glu Gln Leu Glu Glu Leu Ile Ile Pro Ala Glu Lys
20 25 30
Val Ser Glu Glu Glu Leu Leu Glu Ile Arg Lys Leu Lys Glu Glu Ser
35 40 45
Leu Lys Gly Glu His Val Asp Trp Asp Glu Leu Lys Arg Glu Leu Gly
50 55 60
Val
65

<210> 38
<211> 72
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-Af3

<400> 38
Met Lys Val Leu Leu Asp Ile Ile Glu Asp Ile Glu Asn Phe Ile Arg
1 5 10 15
Gln Leu Glu Lys Arg Arg Gly Glu Leu Glu Glu Leu Lys Asp Glu Ile
20 25 30
Leu Ile Phe Ser Asp Ala Glu Phe Ile Asp Ser Ile Gln Arg Gly Leu
35 40 45
Ser Asp Leu Glu Gln Gly Arg Ser Lys Val Cys Ser Asn Leu Glu Glu
50 55 60
Val Lys Lys Leu Phe Glu Asp Ile
65 70

<210> 39
<211> 62
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-Af2

<400> 39
Met Glu Val Ile Gln Ile Ser Lys Asp Glu Leu Glu Glu Ile Ile Glu
1 5 10 15
Arg Lys Phe Lys Glu Val Leu Ile Lys Ala Leu Met Glu Ile Thr Pro
20 25 30
Tyr Val Ser Asp Glu Glu Gln Glu Glu Ile Asp Lys Ile Ala Gly Lys
35 40 45
Pro Asp Glu Tyr Glu Gly Glu Phe Glu Glu Trp His Gly Lys
50 55 60

<210> 40
<211> 57
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-Af4

<400> 40
Met Asp Ile Gln Val Ile Lys Gln Ala Val Arg Glu Val Leu Arg Glu
1 5 10 15
Glu Leu Pro Ser Ile Leu Lys Glu Val Ile Leu Ser Thr Ile Pro Pro
20 25 30
Asp Glu Pro Glu Ala Asp Glu Lys Gln Phe Val Asp Glu Glu Ile Asn
35 40 45
Glu Asp Asp Tyr Val Lys Phe Asp Glu
50 55

<210> 41
<211> 95
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-Hp

<400> 41
Met Pro Asn Thr Thr Asn Lys Asp Tyr Thr Lys Tyr Ser Gln Arg Gln
1 5 10 15
Leu Phe Ser Phe Leu Asn Ser Ile Lys Thr Lys Gln Lys Arg Ala Leu
20 25 30
Glu Lys Leu Lys Glu Ile Gln Ala Gln Lys Gln Arg Ile Lys Lys Ala
35 40 45
Leu Gln Phe Lys Ala Leu Asn Leu Thr Glu Asn Gly Tyr Thr Ile Glu
50 55 60
Glu Glu Arg Glu Ile Leu Ala Arg Ala Lys Asp Thr Lys Asn Arg Leu
65 70 75 80
Cys Phe Lys Ser Ile Glu Asp Phe Lys Lys His Cys Glu Asn Leu
85 90 95

<210> 42
<211> 86
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-syneco

<400> 42
Met Met Arg Ala Phe Glu Val Met Ala Thr Val Lys Asp Ser Lys Gln
1 5 10 15
Leu Leu Leu Asp Ser Asp Leu His Trp Asn Thr Ser Arg Val Lys Val
20 25 30
Ile Ile Leu Glu Ser Asp Glu Leu Ala Ser Lys Gly Ser Glu Phe Asp
35 40 45
Pro Asp Asp Thr Pro Val Glu Glu Ile Lys Val Ser Leu Arg Lys Ala
50 55 60
Leu Glu Glu Tyr Lys Gln Gly Lys Arg Ile Pro Val Glu Asn Met Trp
65 70 75 80

Glu Gly Ile Asp Val Glu
85

<210> 43
<211> 85
<212> PRT
<213> Bacterium

<220>
<223> protein RelB-BT

<400> 43
Met Ala Ile Arg Lys Asp Glu Leu Tyr Arg Leu Ile Asp His Leu Asp
1 5 10 15
Gln Gln Asp Glu Lys Ala Ala Phe Asp Phe Leu Glu Phe Leu Val Gln
20 25 30
Arg Ser Arg Arg Lys Pro Lys Glu Trp Glu Lys Ile Asp Met Ala Asp
35 40 45
Pro Asp His Glu Pro Leu Ser Thr Gln Glu Leu Glu Gln Leu Asn Ser
50 55 60
Glu Glu Gly Tyr Val Ser Gly Glu Asp Ala Lys Arg Glu Phe Gly Leu
65 70 75 80
Gln Ile Asp Leu Pro
85

<210> 44
<211> 1280
<212> DNA
<213> E. coli plasmid P307

<400> 44
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tgatgccatg ccagaatgga cgttcaggtt attccgtcca gttctgctgg caacgcgaga 120
tctcccctgg tatagtgatg ccacagcaaa gcgctcaaac agggataata tgatggaaat 180
caaggctcaa cagttttgtc acatcaacgg ggcggcaagt ccttactgac aacggacaac 240
aaggtatggg cggcgtggcg ggtatcggtt ccacgactga aaagcatcag gggcgcgtgg 300
cggaagcgat ttttgcgaac tgcgcggaac tggataacga ccagcttaac gagatcatcg 360
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acgccccttg tttaattccc gcactacctg gacgttcagg tgattctgtc catctgtaca 480
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ttaaacagtc ttgaaasdst artrbggtgg cctatgccta acattattct cagtgataca 600
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tacgaaaaga tgcttgatgc cctagacgat caggagttgg ttaaasdctg gtagccgaac 780
gcagcaacca accgctgcat gatgtagatc tggataandr bstartrata cctatgaggt 840
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aactgcgtgg gataaaggac tgctacaaaa taaaattacg tgcgtcaggt tttcgcctgg 1020
tctatcaggt gattgacgaa caattaatta tcgctgttgt agctgtgggt aaacgtgndr 1080
agcgcagtga cgtttataat cttgccagcg aaagaatgag ataaaagcaa taaacacaga 1140
aagttactct ggcgttatgg ggtaatgcaa agtatgagtc gtagagggaa ttgcctggat 1200
aattcgccga tggaaagagt ctttcgcagc cttaaaagtg aatggcttcc gaaaggtggt 1260
tatggtgatt ttagccatgc 1280

<210> 45
<211> 1168
<212> DNA
<213> B. thuringiensis

<400> 45
ctcgtttttt ctgttggtac aaacttaatt gattttgaat aatttgtttg taccagtcct 60
ttttgcttag cccagtcaaa ataacgtttg attgaattaa tgcgccggtt aatcgtagaa 120
ggttttagta atcttgtaac ttgcatatgc cctcgatatc gagcaatagt gcgagcggta 180
acttctattg gatgaaaaag agtatcctca gcatgttttc cccacacatt ttcaaaccaa 240
aatacaaaat cttttaaatc actcgtatat tcttttagtg tttttgtatg caaatctcct 300
tcttgagata agctagaaat aaaatcggaa atcaaagatg ttgcttgtat agaaattgtt 360
ttagtggaat gcataaatac ctcctctttt attgacttac rbbtmrnaat tagcggacat 420
gatattttaa tcttatcaat tatgttagcg gacatcaaac atttattttc ccacacttca 480
tgtccactaa tattaattag tggacattrs dstartrbta aaactatctc gaaagtaggt 540
gtaacacatg gctattcgta aagatgaatt gtatcggtta attgatcacc tggatcaaca 600
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taaagaatgg gaaaaaattg atatggcaga tcctgatcat gaaccgctgt ctacacaaga 720
gttagaacag ttaaacagtg aagaaggata tgtatcaggg gaggacgcnd rbstartraa 780
aacgtgaatt cggactacaa attgatttac cataagtccg cggtgaaatt tattgcaaag 840
caagaaaaag ggattcaaaa aagaattgca gaaggattga agggacttct taagattcct 900
cctgaaggag atattaaaag tatgaaaggt tacacagaac tatatcgatt acggattgga 960
acctttcgaa ttttatttga aataaatcat gatgagaaag tcatatacat acaagcaatt 1020
ggaaatcgnd rtggtgacat ctataaataa ggcaaacatg cattttaaa agaaaggtct 1080
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gattctgttc cattgctaca catacccc 1168

<210> 46
<211> 1024
<212> DNA
<213> Methanococcus jannaschii

<400> 46
ccgataccgt tgctggagac atagctggag ctttgaaggc ggagaagctt attttaataa 60
cagatgttga tggaataatg gatgatataa ataatccaga gacgttgcat agaaaattaa 120
cagcttcaga actaaaagaa atgatagaag atggaagaat aaagggaggg atgattccaa 180
aggctgaaag tgccttatat gccttagagc atggagttaa gagcgttcat ataataaatg 240
gaaagattcc tcatgctttg ttgttggaga tatttacaga ggagggtatt gggacgatga 300
taacaagaga ttaaagtttt tatattataa actacttaag aattaaaata startrbmag 360
acaaataagg ggataactat gctcaatata aacaaagaga tagcacaaat agaaactgaa 420
ttgaatgaat tgaaaaaatt gagagatgaa atctctgaaa ggattgaaaa attagaaata 480
aagttattaa aattgaaagc attagctatt ccagaggagg aatttgaaga ggattatgaa 540
gaaattatag aagatgttaa aaaatctctg gataaaaaag agactgtgcc agcagaagag 600
gctttgaand rbmstartrm agaattggga ttattatgaa gtttaacgtt gagatacata 660
aaagagtctt aaaagattta aaggatttgc ctccctcaaa cttaaagaag tttaaagaac 720
taatagaaac attaaaaacc aatcccattc caaaagaaaa atttgatatt aaaagattaa 780
aaggcagtga tgaggtttat agagttagaa ttggaaaatt tagagttcaa tatgttgttt 840
tatgggatga tagaataata ataattagaa ndrmagataa gtagaagaga aggagcttat 900
aaaaatccct aagctattaa aaattctaat ggctacattt ttatatctct tttcttaatt 960
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atag 1024

<210> 47
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer MJ-relE/2CWW was used for the amplification of the relE gene of M. jannaschii from genomic DNA

<400> 47
cccccgaatt cgcatgcgcc attagaat 28

<210> 48
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer MJ-reE/1CW was used for the amplification of the relE gene of M. jannaschii from genomic DNA

<400> 48
ccccccggatc cgagctcgag gctttgaaag aattggg 37

<210> 49
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relB-M.jannCW was used for the PCR-amplification of relB and relE from M. jannashii

<400> 49
ccccggatcc gtcgacgaca aataagggga taactatg 38

<210> 50
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relE-Sp2/cw was used for the PCR-amplification of the gene relESP2 from genomic DNA of S. pneumoniae

<400> 50
ccccggatcc gatgcatgat ttaggcttga ag 32

<210> 51
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relE-Sp2/ccw was used for the PCR-amplification of the gene relESP2 from genomic DNA of S. pneumoniae

<400> 51
ccccgaattc gaatgaaaat ttacttgaaa aaag 34

<210> 52
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relEk12 was used for the PCR-amplification of DNA-fragments comprising genes relEK-12,

relEP307 and relEMj

<400> 52
tgtaatacga ctcactatag ataaggagtt ttataaatgg cgtattttct ggattttg 58

<210> 53
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer P2 was used for the PCR-amplification of DNA-fragments comprising genes relEK-12, relEP307 and relEMj

<400> 53
caccttcggt gcgaaacag 19

<210> 54
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relEP307 was used for the PCR-amplification of DNA-fragments comprising genes relEK-12, relEP307 and relEMj

<400> 54
tgtaatacga ctcactatag ataaggagtt ttataaatga ggtatcaggt aaaattca 58

<210> 55
<211> 20
<212> DNA
<213> Arificial sequence

<220>
<223> Primer P4 was used for the PCR-amplification of DNA-fragments comprising genes relEK-12, relEP307 and relEMj

<400> 55
ctttccatcg gcgaattatc 20

<210> 56
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer relEMj was used for the PCR-amplification of DNA-fragments comprising genes relEK-12, relEP307 and relEMj

<400> 56
tgtaatacga ctcactatag ataaggagtt ttataaatga agtttaacgt tgagatac 58

<210> 57
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer P6 was used for the PCR-amplification of DNA-fragments comprising genes relEK-12, relEP307 and relEMj

<400> 57
atcatggtat cagccgaatc 20

<210> 58
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer S-RelE was used for the PCR-amplification of the RelE coding region from the plasmid pMG223

<400> 58
taggtaccat ggcgtatttt ctgg 24

<210> 59
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer AS-RelE was used for the PCR-amplification of the RelE coding region from the plasmid pMG223

<400> 59
gagaccccac actaccatcg gcg 23

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 33, line 13.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

Name of depositary institution

DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1B
D-38124 Braunschweig
Germany

Date of deposit	Accession Number
30 April 1998	DSM 12157

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

[ ] This sheet was received with the international application

Authorized officer

For International Bureau use only

[X] This sheet was received by the International Bureau on:
13 SEP 1999

Authorized officer

INDICATIONS RELATING TO DEPOSITED MICROORGANISMS
(PCT Rule 12bis)

Additional sheet

In addition to the microorganism indicated on page 33 of the description, the following microorganisms have been deposited with

DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH
Mascheroder Weg 1b, D-38124 Braunschweig, Germany

on the dates and under the accession numbers as stated below:

Accession number	Date of deposit	Description Page No.	Description Line No.
DSM 12158	30 April 1998	33	32
DSM 12159	30 April 1998	35	10
DSM 12160	30 April 1998	34	31
DSM 12161	30 April 1998	33	21

For all of the above-identified deposited microorganisms, the following additional indications apply:

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.